Continuous Culture of Bacillus megaterium

by

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CONTENTS

CONTENTS

I

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II

ABBREVIATIONS AND SYMBOLS

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D = Dilution Rate
μ = Specific Growth Rate
t_d = Doubling Time, i.e. the time required for the mass of organisms to double in weight.
These three factors are related thus:- $\mu = \frac{\ln_e 2}{t_d}$

and when steady state conditions prevail in the chemostat:-

$$\mu = D$$

RNA = Ribonucleic acid
DNA = Deoxyribonucleic acid
PHB = Poly-β-hydroxybutyric acid
CoA = Coenzyme A
DPN & DPNH = Oxidised and Reduced Diphosphopyridine nucleotide.
ATP = Adenosine triphosphate
ADP = Adenosine diphosphate
AMP = Adenosine monophosphate
UTP = Uridine trisphosphate
UDP = Uridine diphosphate
UDPG = Uridine diphosphate glucose
PP = Inorganic pyrophosphate
Pi = Inorganic phosphate
G = Glucose
G6P = Glucose-6-phosphate
G1P = Glucose-1-phosphate

ERRATA

Page 55. Line 19. For 'Pirt and Callow (1956)' read 'Callow and Pirt (1956)'.

Apparatus Section. Where a scale is indicated in the diagrams in this section the values should be one half of the value shown, e.g. Scale $1/_1$ should read $\frac{1}{2}$.

Page 132. Line 10. For 'direct sampling and filtering of the vessel' read 'for direct filtering of the sample from the vessel.

Page 168. Line 12. For 'Holme and Palmstierna (1957)' read 'Holme (1957)'.

The whole spectacle of the chemical and physical transformations carried out by nature evolves out of the operation of a few fundamental laws, the nature of which we are rapidly learning. Living cells conform to the laws of nature, just as inanimate matter, but owing to their chemical complexity, they have resisted man's attempts at a complete understanding of the processes which determine the integration of enzymic activities at the genetic level of organisation.

In the present state of knowledge of living organisms, microorganisms must constitute one of the best materials for enquiry, being without any grossly differentiated structure, and yet showing most of the major phenomena of life. Their single cells contain genetic components which under a variety of external environments, show a remarkable degree of adaptation in the enzymic systems they are capable of producing. Their fast rate of division (compared with cells from higher organisms) is also an added experimental advantage to the investigator. In the world at large, their ability to survive severe physical conditions and almost certainly periodic if not continual starvation, must indicate special mechanisms to regulate

cellular metabolism and to provide some reserve of energy-rich material. Indeed these would seem essential prerequisities in the competition for survival. That we can learn many lessons from microorganisms surely widens the territory in which it is possible to operate with microbiological methods, enhancing the feeling of the 'unity of science' and facilitating cross-fertilisation between different disciplines. A scientist working with such useful research tools as bacteria must always be aware that the level of significance of his results, i.e. the depth to which he can penetrate into a problem, is severely limited by the methods he can devise. In this thesis it is hoped to show that a method of culturing bacteria, by a continuous process which was developed independently by Monod and by Novick and Szilard, is capable of extending our knowledge of the function of some components of the microbial cell which are left ambiguous by the more normal method of batch culture.

Attempts to culture microorganisms continuously have been recorded over the last thirty years (Rogers and Whittier, 1930 and Unger et al. 1942, and Myers and Clark, 1944). However, the application of continuous culture to fermentation processes and experimental micro-

biology has, as already mentioned, only been systematically developed over the past thirteen years. Moreover, the earlier authors never appreciated that this practical approach is associated with the more profound study of the physiology, biochemistry and genetics of microorganisms. They did not consider continuous culture as a new and basically different method, and as one which better relates the reproductive capacity of microorganisms than the commonly used static method.

The experimental laboratory technique, developed over recent years has given a successful working apparatus while offering at the same time a general theory of continuous culture. Basically, a continuous culture (chemostat) apparatus consists of a culture vessel in which the microorganisms are grown. This culture vessel is thoroughly stirred, while fresh medium is continuously fed into it at a constant and accurately metered rate. A constant level overflow keeps the volume of the culture in the growth vessel steady and allows the culture to leave at the same rate as fresh medium flows into it. The successful application of the theory behind this process has shown that it is necessary to devote considerable attention to a knowledge of those physiological processes which represent

the basis of the respective species of cell under culture, whether they be procaryotic or eucaryotic. The basic feature is that it gives a considerable measure of control of the physiological state of the microorganisms used. Initially, in a batch culture, the organisms grow in an excess of all nutrients at a rate specific for that medium, substrate continually decreasing until it reaches a level that reduces the growth rate below that specific for the medium; finally both fall to zero. In the chemostat however, the continued steady addition of fresh medium fixes the limiting nutrient concentration, and hence the growth rate. Two papers published in 1950 contained these basic facts, first Monod described the 'Bactogen' and later Novick and Szilard described the 'Chemostat', both being similar in function. The name 'Chemostat' has subsequently been generally adopted.

A second type of continuous culture apparatus is also possible, being known as the turbidostat, (Bryson, 1952). In the chemostat the dilution rate, D (ratio of flow of medium to culture vessel volume) and hence the concentration of the limiting nutrient is set at a predetermined level; in the turbidostat the bacterial concentration is held at a fixed value by the intermittent addition of fresh medium

to keep a constant turbidity. Thus, in a turbidostat the cells are always growing in an excess of nutrients, the growth rate being controlled by an internal cellular system, the maximum growth rate afforded by the medium being the dilution rate for this type of continuous system; chemostat cells are grown in an externally and manually controlled system and turbidostat cells are grown in an internally controlled system or, as described by some authors, in unlimited "balanced" growth. Therefore, the two are complementary to each other, the chemostat working at lower growth rates and the turbidostat working at the maximal growth rate.

The necessary theory of reactions for continuous flow systems was detailed by Denbigh (1944, 1951). His treatment of the continuous flow stirred tank reactor is the basis of many continuous chemical processes including continuous culture.

Comparison of Batch and Continuous Culture

It would seem important at this stage, to compare, to the best of our present day knowledge, a microbial population cultivated in a continuous culture with the broad spectrum of material that may arise from a batch culture as much of the material reviewed in this thesis

is based on batch culture techniques.

In a typical laboratory batch culture, micro-organisms are inoculated into a sterile flask containing a large excess of all possible nutrients; throughout the many recognised stages of development, lag, exponential and stationary and the subdivisions of each, changes occur in the enzymic, morphological and chemical properties of the cell and in the composition of the medium. Two stages of the batch culture, namely the lag-phase and the stationary phase, find no comparison in the continuous culture. Although in the world at large it may be untrue, these developmental changes in the laboratory flask are artifacts; the lag phase appears because a dormant culture must adapt itself to regular multiplication; the stationary phase appears only because of complete exhaustion of one, or more than one, nutrient, or because an extracellular product(s) has become bacteriocidal or at least bacterio-static. These states are not a prerequisite, as stages in a life cycle, as has been proved by the continuous culture technique over infinite periods of vegetative growth of many differing types of organisms (Herbert, Elsworth and Telling, 1956, Herbert, 1958, Malek, 1958, Postgate & Hunter, 1962). To enable a comparison of the two cultures, only those con-

ditions which allow division to proceed can be called physiological, as everything else appears to be an artifact of inadequate conditions for multiplication. A rejection of this view might be based on the developmental changes observed in the stationary phase, e.g. sporulation and resting forms. These same organisms can, however, be kept indefinitely in the vegetative form in continuous culture.

Assuming then, that the organisms to be compared are dividing, however slowly, it is probably best to list their reactions according to the conditions that allow division, and according to their reactions to changes in the growth medium.

1. In a batch culture, cells in the exponential phase are growing in an excess of all nutrients, when an internal system similar to that operating in a turbidostat limits the growth rate. At the end of the exponential phase and depending on the complexity of the medium, shortage of an essential nutrient will more or less rapidly bring growth to a halt. Continuously growing cells in a chemostat are limited, at all times, from faster division by limitation of an essential nutrient. Turbidostat conditions will be discussed later as will some of the effects of the low concentration of the limiting growth fraction in relation to repression and derepression of enzyme systems.

2. Some properties of an organism are a function of the composition of the medium at the moment of definition and will change according to changes in the latter, e.g. Schaechter, Maaloe and Kjeldgaard (1958) found that the number of nuclei per cell, the cell size, and the cellular content of DNA and RNA were exponential functions of the growth rate afforded by the medium. These experiments were carried out in a vessel resembling, in its working principals, a turbidostat, where all nutrients are present in excess and where cell concentrations are very low, in order that toxic products of metabolism accumulate to no great extent. However, Kjeldgaard, Maaloe and Schaechter (1958) also established in these experiments that, following transfer of a culture to a medium affording a higher specific growth rate, there is a dissociation of the basic synthetic activities; the rate of synthesis of RNA reaching a maximum value for that medium in seconds, cell material in 5 min., DNA within 20 min., the number of nuclei per cell within 50 min., and cell division in 70 min. Therefore, in a batch culture, where the medium is changing fractionally all the time, some properties of the organism are not a function of the actual composition at the moment of definition, but are a function of its composition as it

was some time before. These properties are determined by the history of the process of development and are a function of elapsed time, there being a 'lag' between the inducement and the response. Quantitatively then, there is no comparison between a batch culture in which conditions change all the time and the organisms havea specific history, and a continuous culture when conditions are stable and the organism has no history.

Properties which undergo 'lag' type changes, i.e. the finite time, which is not specified and arbitary, that passes between a certain external stimulus, and the reaction of the cell to that stimulus, are important in comparing batch and continuous cultures. The delayed reaction is the sign of a series of alterations involving one property, which may be appearing or disappearing, and which we have chosen to follow because it will manifest itself under the given conditions. These properties may not show themselves at the given time, but will only attain significance in the further development of the culture, e.g. the acquisition of a property may persist throughout a batch culture and only manifest itself in the final phase. Many changes may be due to one stimulus but with 'lags' of different duration. Some examples of stimuli which may induce these changes are inoculation, entire exhaustion of a

medium component and accumulation of a critical level of an end product of metabolism. Thus we might say that the longer the 'lag' the more reactions are involved in the induced change.

It is usually assumed that exponential-phase batch and continuous cultured organisms are identical, or at best very similar, if the specific growth rates are equal. This is most certainly so between cells in the middle exponential phase and turbidostat grown cells, and between late exponential phase cells and chemostat grown cells near the minimum doubling time for the medium. This ignores the fact that both types of batch culture cells have a specific history of development. Some properties then are definitely functions of the specific growth rate and each of these must depend on the medium composition. On the other hand, a property may depend on the medium composition but not the specific growth rate, e.g. during growth of yeast, the formation of ethanol is limited by the concentration of sucrose whereas the formation of cell substance is limited by the concentration of the nitrogen source (Maxon, 1953).

In a medium containing more than one carbon source it is likely that there will be a successive exhaustion

of the carbon sources, i.e. the diauxic phenomenon will occur. Also, there may be product formation due to growth exceeding oxidative metabolism, the products being consumed after the growth rate has been reduced. A pertinent example is the formation of acetic acid by Bacillus megaterium when grown in batch culture on glucose based medium (Nakata and Halvorson, 1960). It was found that there were two oxygen demand peaks, one corresponding to glucose utilisation and maximum growth and division, and the second, after all glucose had disappeared corresponding to oxidation of acetic acid associated with growth, but no division. Considering a single stage continuous culture with carbon limitation on a mixed carbon source, will the diauxic phenomenon occur here, especially when the organism is being forced to grow at slow rates? This type of behaviour is fundamentally different from that in batch culture, where one carbon substrate is reckoned to repress metabolism of another. In a chemostat, extracellular and hence intracellular concentrations of the repressor substrate may never be enough to saturate all the active enzyme sites and hence effect repression of the other substrates. The dilution rate, i.e. the factor controlling the repressor

concentration, then assumes paramount importance as demonstrated by Gorini (1960). In batch culture enzymic systems adapt to each successive nutrient.

Properties which exemplify themselves by a 'lag' in growth during batch culture may be demonstrated in continuous culture. By altering the pH every few minutes such that a sinusoidal plot of the value was obtained, Fuld, Mateles and Kusmierek (1961) found that the oscillations of the population resulted from the growth rate being a 'lagged' function of the pH. No steady state was obtained. This was a case in which the 'lag' manifested itself in an easily measurable fashion, but other properties may give stable oscillations during the so-called steady state without affecting the growth rate, because they are independent of it.

Chemical Composition and Morphology

The chemical composition of microbial cells reflects the type of environment in which the cell has been produced. Changes in the chemical and physiochemical nature of the enviroment have the most significant effects on the composition of the organism both from the qualitative (whether anconstituent is present or not) and quantitative (amount of component present) viewpoint. A

notable example described in an other section are poly-glucose storage compounds (Holme, 1957). The continuous culture apparatus gives us a hitherto unachieved control of the environment and hence the growth rate, the two most important factors in cellular composition. This was well illustrated by Gorini (1960) who studied the mechanism governing the regulation of enzymic levels. He found that a mutant of Escherichia coli, with a block in an intermediate step in arginine synthesis, had a barely detectable ornithine transcarbamylase action when grown in the presence of arginine. Arginine was known to repress this latter step in arginine synthesis. However, when cells were grown on limiting arginine at DO. 46 $hr.^{-1}$ and below in a chemostat, the steady state level of ornithine transcarbamylase was 25 to 50 times larger than those levels found in the wild type growing in minimal medium. Increased dilution rates, giving a higher concentration of arginine, led to a decrease in the rate of enzyme produced.

Schaechter et al. (1958) have carried out extensive studies, using Salmonella typhimurium, in a vessel of the repeated dilution type (Turbidostat). Many different types of complex and simple growth media were used giving doubling times from 22 to 97 min. The following cellular

components were determined in each culture; dry weight, cell count, RNA, DNA, and the number of nuclei per cell. Using the data recalculated by Herbert (1961) their results show that the RNA content of the cell, expressed as a percentage of the dry weight, increases with increasing growth rate, while the percentage of DNA decreases. The average number of nuclei per cell in this organism also increases exponentially as a function of the growth rate, at nearly the same rate as the DNA per cell, so the weight of DNA per nucleus shows very little change with growth rate, although RNA per nucleus increases considerably. Herbert (1958) using a chemostat, has obtained nearly comparable results. Over a wide range of growth rates he studied cell mass, RNA, DNA, and protein contents of Aerobacter aerogenes grown in glycerol limiting, ammonium salts medium, and B. megaterium, grown in a casein hydrolysate mannitol limiting medium. Cell mass, RNA and DNA gave linear plots with logarthmic ordinates against growth rate in the range of doubling times used by Schaechter et al. (1958), but fell off at lower rates which are only attainable in the chemostat. When Herbert cultured Staphylococcus aureus continuously, similar results were obtained except for cell mass, which is apparently constant for cocci regardless of the growth rate.

Pirt and Callow (1959) studied the influence of pH on the morphology of Penicillium chrysogenum in a chemostat under glucose limitation at a low dilution rate (0.05 $hr.^{-1}$). Filamentous growth was achieved at pH 6, but on raising the pH to 7 many involution forms occurred with a general thickening of the hyphae. It was suggested that the resistance of the cell walls decreased because of increase of pH. Pellet formation was also noted to be dependent on pH. Pirt, Thackeray and Harris-Smith (1961) also show how important environmental control is in vaccine production. Culturing Pasteurella pestis at a low growth rate (0.1 $hr.^{-1}$). they followed quantitatively the yield of three different antigens over a range of temperature and pH, and found that production of some antigens could be induced only during stabilisation at 37° of a culture originally maintained at 28°. However, against this, selection against virulent types occurred at 37° but not at 28°. These authors suggest that by using a two stage process, with the growth stage at 28°, the cells would be kept in the virulent phase, and then by allowing the organisms to pass to the second stage at a higher temperature, material of the desired composition could be obtained.

Richmond and Maaloe (1962) cultured Salmonella

typhimurium continuously in a vessel giving unrestricted balanced growth. Cells grown in glucose and compounds related to it metabolically were white and had relatively low contents of cytochrome and inorganic iron, while cells grown in substances related to the Krebs cycle were pink and had relatively high cytochrome and iron contents. The cytochrome content of a strictly aerobic pseudomonad under conditions of oxygen and also succinate limitation were investigated by Rosenberger and Kogut (1958). These authors observed that the oxygen-limited organism had twice the cytochrome content of the succinate-limited ones at the same growth rate.

The latter two examples show that, while different media may give similar growth rates and similar cellular contents of RNA and DNA (Herbert, 1958 and Schaechter et al. 1958), inducible enzyme systems will react to local conditions and individual limiting nutrients in a quantitatively different manner.

Yield Measurements

The maximum output of cells in a continuous culture may be deduced from measurements of the doubling time of cells in a batch culture (Herbert et al. 1956). Good agreement has been obtained by these authors and

Herbert (1958) for A. aerogenes, B. megaterium, Torula utilis and Staphylococcus aureus for carbon limitation. Confirmation has also been obtained for nitrogen limitation by Holme, (1957) who cultured E. coli, and by Dawson (1960) with an osmophilic yeast, Saccharomyces rouxii.

Postgate and Hunter (1962), cultivating A. aerogenes continuously with limiting carbon, oxygen, nitrogen, sulphate, phosphate, or magnesium, have found that some deficiences, namely of magnesium and of phosphate, apparently do not follow any previously reported yield pattern. The growth yield drops linearly with increasing dilution rate and the output of organisms is nearly constant over the range of dilution rates tested. Such behaviour is difficult to account for, a possible explanation may be that the organism has a high Michaelis constant for the limiting nutrient. Whether the kinetics of uptake of magnesium, or of phosphate through a permease system are involved, or whether they are the sum of the kinetics of the internal enzymic activities, is unknown. Regardless of which system is involved it would seem that a high concentration of either Mg^{2+} or of PO_4^{3-} is necessary to drive the reation rates

faster to give increased growth rates. The use of tracer techniques should easily establish whether the concentration gradient is inside the cell or at the membrane. Postgate and Hunter (1962) also found that carbon (glycerol) and magnesium limitation gave lower viabilities at low growth rates, namely 70% viable at D 0.1 $hr.^{-1}$ compared with 90% for nitrogen, phosphorus and sulphate.

Contois (1959) has studied yields of A. aerogenes in both batch and continuous culture. His results for glucose, succinate or nitrogen limitation show that the specific growth rate is not only a function of the limiting nutrient but also of the population density. This means that in two chemostats with populations at different densities, growing under the same limiting nutrient at similar growth rates, the concentration of this limiting nutrient is different in each vessel. However, the theories of Monod (1950), Novick and Szilard (1950a) and Herbert et al. (1956) show that for a given limiting substrate concentration, regardless of population density, the growth rate is fixed. The correction factor is not large, but does seem to exist.

Carbon limitation has been universally tested, the general results being that the cell yield does not follow

the predicted pattern, but decreases at low growth rates. As pointed out by Herbert (1958) the most probable explanation of these results is that, in addition to the anabolic metabolism, the organisms also have an endogenous catabolism, by which cell substance is oxidised to carbon dioxide. At slow growth rates, this endogenous metabolism becomes proportionally more important compared with the anabolic metabolism, and the cell yield decreases.

Rosenberger and Elsden (1960) continuously cultured Streptococcus faecalis and measured growth yields in relation to anaerobic energy metabolism with glucose or tryptophane limitation. They found that the rate of glucose catabolism with tryptophane limitation was independent of the growth rate. This can only mean that in Strep. faecalis the rate of catabolism is not controlled by the rate of cell synthesis.

Growing Salmonella typhimurium by the unrestricted balanced growth method, Maaløe and Richmond (1962) found that, in a simple defined medium containing proline or glutamate as the sole carbon source, the organism divided at 0.7 and 0.9 doublings per hour respectively. Glutamic acid is readily metabolised via the Kreb's cycle, while proline is converted to glutamic acid

by a series of inducible enzymes. The experiments showed that, even in the fully induced state, the rate of conversion of proline was limiting in the sense that the cells would have grown faster had they been supplied glutamic acid. Whether, during balanced growth in the proline medium, glutamic acid was actually limiting is another question. At least two answers are possible. First, both amino acids give comparable amounts of enzymic material, but the need for synthesising the proline-degrading enzymes, in addition to the other enzymes, reduces the growth rate in the proline medium; and second, the efficiency of the degrading enzymes is so low that it is impossible to establish a balanced situation in which the enzymes converting glutamic acid to cell material are saturated. In the latter case the glutamic acid concentration could be said to be limiting. Thus some cases of balanced growth may be more akin to conditions in a chemostat.

Genetic Variation

Periodic selection occurs in all cultures of bacteria whether artificially grown by man or growing naturally (e.g. in the soil). The selective pressures may only induce some different enzymic expression or select an organism genetically different. Often it is not possible to

reveal the nature of the former type of change, the observed characteristics of the bacterial culture being maintained. A type of selective pressure inducing a genetical change in a continuous culture was described by Powell (1956). He found that a continuous culture selects heavily against organisms of unusually long generation time. This is one argument against the idea that a continuous culture is ageless. As time passes, this type of mutation will occur. Those mutants that grow faster than the original parent type will therefore grow more rapidly than the wash out rate, and will thereby increase the number in the population. In an externally controlled system of the chemostat type the faster strain will displace the original population. This will occur as the faster strain brings about a reduction in the concentration of the controlling growth factor to a level where the growth rate of the faster strain equals the washing out rate. Under these conditions the growth rate of the original strain will be less than the flow rate, resulting in the washout of the parent strain.

An example of this type of genetic variation was provided by Novick and Szilard (1950b) who were studying the linear increase in the frequency of bacteriophage T_5 resistant mutants in their strain of E. coli B.

growing in a chemostat. As there is a spontaneous mutation to T_5 resistance in this strain a linear increase in the T_5 resistant mutants was expected, the slope of the line being proportional to the mutation rate. The expected equilibrium that should have been obtained was never established, instead there was a sporadic decrease followed again by an increase in the number of T_5 resistant mutants. Once the faster strain is established, and if the rate of mutation to T_5 resistance is the same as in the initial strain, a linear rise in the number of T_5 resistant mutants should again be observed. Therefore, observation of the number of T_5 resistant mutants provided a way of detecting such population changes when observation of the total population gave no indication. The authors reported more than 10 or 11 transitions from slower growing to faster growing strains in approximately 500 generations at low concentrations of the controlling growth factor.

In an experiment with a turbidostat, Cocito and Bryson (1958) demonstrated that mutants of E. coli B. resistant to phage T_3 were able to outgrow their phage-sensitive parents at an exponential rate in the vessel.

They discovered that the parent strain was liberating a substance, tantatively identified as a colicine which was inhibitory to the parent. The mutant strain apparently predominated after approximately 40 hr. The result of this experiment has a certain resemblence to that of Novick and Szilard (1950 b) except that the mutant outgrew the parent exponentially, meaning in this case that its growth rate, regardless of the mutation rate, was faster than the parent. The roles may have been reversed in the former example in that a colicine produced by the parent suppressed the mutant. Novick and Szilard reported that the mutants rate of decline was erratic.

In turbidostat experiments Cocito and Vogel (1958) designed tests to detect possible selection against dispensable enzyme formation using the acetylornithinase system in E. coli. L-ornithine is capable of repressing acetylornithinase synthesis. Thus when this amino acid is added to the medium the enzyme is not required for the production of ornithine. After 3 - 7 days growth in the turbidostat in the presence of the repressor ornithine, the organisms isolated by subculture gave enzyme levels as low as one tenth the normal level, the modification

evidently being a heritable factor.

Formal, Baron and Spilman (1956) continuously cultured Salmonella typhi with nitrogen as the limiting growth factor. No decrease in immunizing capacity or virulence was observed, but after two weeks cultivation the strain showed a change of surface antigens, detectable by serological techniques. Holme and Edebo (1961), growing Salmonella typhimurium continuously in a synthetic medium at different dilution rates, observed no departure from smooth colonies in daily plate counts. They claimed that rough colonies could easily be isolated from aged batch cultures of the same strain.

By comparing serial transfer with continuous culture of Clostridium saccharobutylicum, Finn and Nowry (1959) found no decrease in glucose utilisation and solvent production during 650 generations in 2 weeks of continuous growth; however, after 19 generations comprising 4 serial transfers, the cells had retained practically no capacity for solvent production.

Jerusalimskij (1958), continuously culturing Clostridium acetobutylicum for 200 days i.e. 4,000 vegetative generations corresponding to 400 - 600 transfers on normal media, found that the bacteria fully maintained

their normal fermenting ability and retained their ability to sporulate. In the presence of an increasing amount of butanol, the culture of Cl. acetobutylicium changed, in that it became more resistant to this toxic material. The original culture withstood only 0.8% butanol, but after 21 days of growth in the presence of 0.6% butanol it withstood up to 1%. The increased resistance did not remain after passing through one spore cycle. However, in the course of further adaptation the resistance to butanol reached 2.5% and there remained constant. The level of resistance was then transferred through the sporing form to further generations, showing a hereditary character. This type of change, apparently written into the genetic character after an unspecified time, poses the question, was the change a sudden all or none phenomenon? Therefore, it might seem possible to promote a stable genetical change by the choice of suitable selection pressures in a continuous culture. Degeneration of a particular property, i.e. loss of ability to produce a desired product, whether intracellular or extracellular, is one manifestation of this type of change.

Postgate and Hunter (1962) noticed alterations of

at least three characteristics of Aerobacter aerogenes after long periods of continuous culture, namely: - 1. A doubling in length and a wider scatter of mean lengths from the parent culture. 2. The minimum mean generation time on transfer to batch culture was slower, 5 min., compared with 44 min. for the original culture. 3. The organisms developed a long lag when plated on tryptic meat agar upon which up to 70% did not divide. The slower generation time after continuous culture would appear to contradict Powell's statement that continuous cultures should select faster growing strains. However, the predominance of the slower growing strain is only the selection of the strain best suited to grow at the time of sampling.

Cellular Constituents as Storage Products

Biological economy, based as it is on the competition for food, necessitates that most living organisms be equipped with the means of storing any nutrients available in excess of immediate requirements for use when the external food sources fail. It has long been recognised and proved that such materials as starch, glycogen and lipid form reserves of carbon and energy in the organisms of the plant and animal kingdom, but much confirmation has still to be received of their role in bacteria. The stored

materials may either form localised deposits such as glycogen or lipid granules, or be dispersed throughout the cytoplasm. Generally, storage compounds are of high molecular weight, low in solubility, and because of their physical properties, such as low osmotic pressure and absence of pH effects, exert little influence on cellular activities. They are synthesised from available nutrients, usually at the expense of energy, and are not just accumulations of particular substances present in the food. Most important of all, a survey of what are believed to be reserve materials shows they are potentially rich in energy provided they can be fully oxidised. Materials like lipids and polysaccharides, lacking in nitrogen, can therefore act primarily as reserves of energy, and also intermediates, being there to tide the organisms over periods of nutritional hardship. Possession of these reserve nutrients must endow the owner with greater chances of survival than any lacking in such reserves.

Present day knowledge still awaits unequivocal proof that the endogenous metabolism in bacteria furnishes the energy required to maintain bacterial viability. Energy is required for osmotic regulation, maintenance of intra-

cellular pH and motility, and anabolic mechanisms such as turnover of proteins and nucleic acids (Dawes and Ribbons, 1962). In the absence of exogenous nutrients, this energy of maintenance must be derived from endogenous sources, but if it can be shown that endogenous respiration is primarily due to the breakdown of a compound with no other function in the cell, then this may indeed be a true storage compound. Some difficulty lies in defining materials which function only as exhaustible nutrient reserves, and other compounds which have an essential role, but which might, under the stress of prolonged starvation, be utilised to provide energy and intermediate metabolites. It should be remembered that a bacterial cell may contain more than 50% protein and up to 30% RNA. When a cell stops actively dividing it is not known what proportion of this material becomes redundant and suitable for degeneration. The evidence shows that bacteria differ considerably in their substrates for endogenous respiration and that, although many of these may be typical storage compounds, this is not always the case. Thus Dawes and Holms (1958) have shown that Sarcina lutea will utilise the internal amino acid pool for endogenous respiration.

Materials acting as Reserve Stores:

The materials most commonly referred to as storage compounds in microorganisms are as follows:-

1. Polysaccharide: Chiefly the polyglucose type, occurring intracellularly either as granules or dispersed in the cytoplasm. It is most unlikely that extracellular polysaccharide can be utilised by a cell due to separation by the cytoplasmic membrane of any products of enzymic hydrolysis. Even if it was a storage mechanism, it would be a very wasteful process.

2. Lipid suitable for storage functions, as opposed to structural lipid, is usually found in lipid granules. These granules have the property of staining with fat soluble dyes such as Sudan Black B and occur in many microorganisms. The most common constituent of bacterial lipid granules is poly-β-hydroxybutyric acid (PHB), but it is possible that some bacteria, like yeasts, store more conventional lipids such as triglycerides. 3. Polyphosphate: Polyphosphate material has been shown to act as a phosphate store and possibly as a source of high energy phosphate.

Proof of Storage Function:

Proof can come from clear cut demonstration that substances are utilised by the cell in absence of exogenous food, primarily for the provision of energy, and also, but not necessarily, for intermediates. Another possible reason for the presence of lipids and carbohydrates is that

they are formed on sugar rich media, and represent an attempt to render harmless, end products of metabolism which might otherwise accumulate too rapidly and prove toxic. They would only be of temporary value, as unlimited intracellular accumulation would eventually burst the cell. This function might be indicated if the complexes were later broken down to soluble units without production of utilisable energy and thus be excreted by the cell. Therefore, it is clear that the nature of the catabolism of the material is a final test of its function. If it is oxidised, it is reasonable to suppose that the energy produced is utilisable, though absolute proof would be difficult to obtain. That a stored material has been used for growth has been proved by several authors (Holme and Palmstierna, 1956 c, Doudoroff and Stanier, 1959). However, storage compounds rarely accumulate to more than 50% of the dry weight, storage space being the limiting factor; also the proportion of a carbon storage material, or even a carbon nutrient, that is assimilated is low, perhaps 40% incorporation, the rest providing energy. Thus maximal storage can only give a fractional increase in growth.

Although final proof of the function of a stored material may be elusive, it is often easy to demonstrate

quantitative variability of an endogenous component. Much evidence has now accumulated that the mechanism underlying the production of a reserve material is that any nutritional deficiency which independently limits growth may promote accumulation. Now it has already been shown that in a chemostat, growth can be dissociated from other cellular activities. Thus it can be shown that by growing an organism at various growth rates with the same nutrient deficiency, whether a cellular component is dependent, or is partially or totally independent of growth rate, and by comparing different nutrient deficiencies, whether a particular nutrient plays an active role in synthesis of the cellular component. If the cellular component is suspected of being a storage reserve, then obviously it will be interesting to see if the organism can break it down under conditions of starvation.

In order that a metabolised product be of value to the cell as a nutrient reserve, it seems probable that it should remain in the cell. There are substances which may be considered non-functional, which are produced under conditions of excess utilisable carbohydrate. These substances or overflow products, mainly organic acids, are produced as a result of incomplete oxidation of the carbohydrate which had been present in excess amount. These products

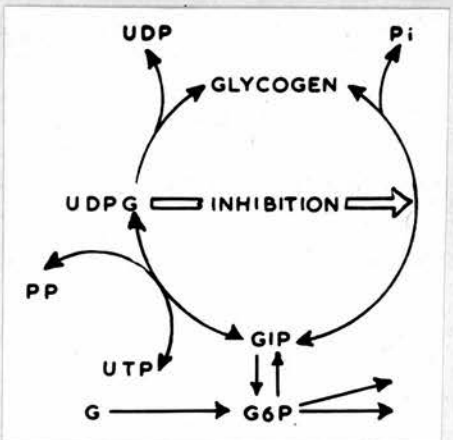
are described by Foster (1947) as being no different from complexes such as lipid and polysaccharide, since, he points out, either group may be used for growth under appropriate conditions. However, these are products of overflow metabolism, since they diffuse across the cytoplasmic membrane and will almost certainly be lost to the cell (except in a batch culture flask) as distinct from an intracellular storage product. An overflow product requires no energy for its synthesis, it being the result of a rapid breakdown of carbohydrate into intermediate products with which subsequent enzyme systems cannot deal. A storage compound however, will require the expenditure of free energy, as any polymeric component of the cell will require energy spent on its synthesis. Furthermore, most overflow products are pathological, being only produced in media where there is a large excess of carbohydrate per cell. Typical storage compounds, while being produced in rich media, are still formed to an appreciable extent even in media poor in energy sources.

Intracellular Polysaccharide

This type of material accumulates in the cytoplasm either as granules, or in a soluble form, being usually synthesised in media containing excess of a carbon and energy

source, such as carbohydrate; maximal accumulation is achieved in the stationary phase of nitrogen-limited batch cultures (Holme and Palmstierna, 1955) and at low growth rates in nitrogen-limited continuous cultures (Herbert, 1961, Holme, 1957). This intracellular substance is of the polyglucose type, resembling glycogen in many of its chemical properties. Materials of this type have been described in a variety of organisms, including, B. megaterium (Barry et al. 1953) E. coli B, (Palmstierna, 1956) Agrobacterium tumefaciens (Madsen, 1961), T. utilis (Herbert, 1961) Rhodospirillum rubrum (Stanier et al. 1959).

Madsen (1961, 1963) has presented evidence for the mechanism of control of glycogen synthesis in cells of Agrobacterium tumefaciens. As well has having an inhibitory effect on the action of phosphorylase, UDPG is the substrate for glycogen synthetase thus providing a basis for the control of glycogen metabolism as shown:-



The level of UDPG and glycogen in batch culture shows that whenever the UDPG level rises (presumed to come from RNA breakdown) continuous synthesis of glycogen would continue for approximately 2 hr. and then the rate would fall, while the accumulated glycogen level remained constant. The fall in the rate of synthesis was due apparently to a drop in the level of UDPG. If fresh ammonium salts were added during the 2 hr. the UDPG level dropped quickly, division started, and the glycogen level dropped after each succeeding division. Whether the glycogen was diluted by cells dividing or was utilised during growth, or if further synthesis was inhibited is unknown. Holme and Palmstierna (1956a & b) have also studied glycogen formation in E. coli B, under a variety of nutrient limitations in batch culture, and with nitrogen limitation in continuous cultures (Holme, 1957). Large amounts of intracellular polysaccharide were accumulated with nitrogen deficiency during the stationary phase of batch culture, but little was formed during the corresponding period of sulphate, phosphate and carbon deficiency, although during exponential phase growth, there were small amounts formed. Even during the stationary phase

of phosphate and sulphate deficiency when glucose was in excess, little additional glycogen was synthesised. The authors did not, however, investigate respiration rates to see if the whole metabolism was retarded, or just glycogen metabolism. Nitrogen-limited continuous culture studies showed that the glycogen content of the cells increased at low dilution rates. However, the rate of glycogen synthesis, glucose being in excess, dropped slightly with increasing growth rate, from about 40 µg/mg dry wt./hr. at D 0.2 $hr.^{-1}$ to 35 µg/mg dry wt./hr. at D 0.8 $hr.^{-1}$. The synthesis rate below D 0.2 $hr.^{-1}$ fell off very rapidly to 20 µg/mg dry wt./hr. at D 0.1 $hr.^{-1}$. Herbert (1961), growing T. utilis under nitrogen limitation, has obtained very similar results. At this point the level of some nitrogen containing compound must have reached a critical level and this is interesting in relation to Madsen's views on glycogen synthesis. It would seem that the NH_4^+ ion concentration is at least indirectly concerned. The very reduced amount of glycogen in either phosphate or especially sulphate deficient cells is unexpected. However, it must be remembered that high concentrations of NH_4^+ ions were present.

Holme and Palmstierna (1956c) did not test whether the glycogen formed was a substrate for endogenous meta-

bolism, but they did obtain evidence that glycogen can serve as a source of carbon for the synthesis of nitrogenous materials. Stationary phase cells from nitrogen deficient cultures were allowed to assimilate uniformly labelled ^{14}C-glucose into glycogen. When the cells were subsequently incubated with a source of nitrogen, but without a carbon source, ^{14}C flowed from the glycogen to the protein fraction. A breakdown of some 25% of the total glycogen sufficed for the label to be lost, indicating that the last formed glycogen is the first to be degraded. Therefore, in these experiments glycogen did fulfil the criteria of an energy reserve substance as defined by Wilkinson (1959).

Ribbons and Dawes (1963) have studied the endogenous reserves of stationary phase Sarcina lutea grown on peptone and glucose-peptone media. This organism was found to use the free amino acid pool as the primary source of endogenous respiration during starvation. However, grown under nitrogen limitation with glucose in excess, it will store a polyglucose compound, which will act as an endogenous reserve along with the amino acid pool. The same authors also studied the endogenous

respiration of E. coli grown in batch cultures. For this organism it was found that the endogenous Qo_2 was directly related to the cellular content of polyglucose. Thus stationary phase cells, harvested from glucose-ammonium salts, glucose-tryptone, and tryptone alone were compared for glycogen utilisation and ammonia release. The two former media yielded stationary phase cells containing glycogen which was initially utilised as an endogenous substrate; ammonia release followed glycogen depletion. Tryptone-grown cells released ammonia immediately on starvation but contained no glycogen. Thus the chemical composition of the growth medium influences not only the cellular composition but also how the endogenous reserves are utilised.

This last point is well supported by the observations of Strange, Dark and Ness (1961) who have carried out exhaustive investigations on the survival of stationary phase, washed cells of A. aerogenes in sodium chloride-phosphate buffer solutions. The cellular composition differed according to the carbon source of the growth media which contained one of the following; mannitol-ammonium salts, tryptone-glucose, or tryptic meat broth. Many changes, with little loss in viability, occured in the chemical composition

of washed suspensions of the organism on prolonged aeration (140 hours). Tryptone-glucose grown cells had a high carbohydrate content, 21%, and glycogen was depleted during the initial 25 hours of incubation along with a small decrease in protein and no change in RNA content. After glycogen had been utilised, further degradation of protein occurred and, to a lesser extent, RNA degradation.

These latter changes were accompanied by release into the medium of ammonia and UV - absorbing material. Cells from tryptic meat broth and the defined medium, which contained much less (4 - 6%) carbohydrate, degraded their protein and RNA with little change in the carbohydrate content.

Postgate and Hunter (1962) have carried out similar investigations on the survival of continuously grown A. aerogenes in sodium chloride-phosphate buffer solutions. Cells were harvested from a series of media deficient in one of the following, carbon, phosphate, magnesium, sulphate, or oxygen. RNA was the first material to be degraded, followed after a lag by protein; intracellular polysaccharide appeared to be unaltered. However, all the cells,

even those grown under nitrogen limitation, contained low (3 - 4%) amounts of polysaccharide, much of which may have been structural components as the method used for analyses would not detect the difference. The medium used by Postgate and Hunter (1962) would perhaps not favour polysaccharide production, glycerol being used as the carbon source; although each element undoubtedly limited growth, there was insufficient glycerol to give an excess, a fact not sufficiently stressed by the authors, as almost certainly all the glycerol would be oxidised at the lower growth rates.

Therefore, it would seem that whenever intracellular polyglucose compounds occur there is good reason to suspect that they may act as reserves of energy and possibly intermediate metabolites.

Overflow Metabolism

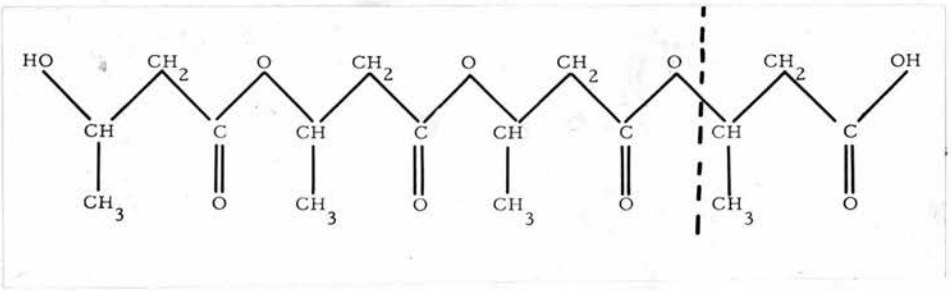
In conjunction with studies on glycogen in E. coli B, Holme (1957) also investigated the production of extracellular materials during nitrogen limitation in continuous culture. Holme discovered production of two 2-oxoacids, namely pyruvate and 2-oxoglutarate, and also a strong UV-absorbtion in the effluent medium which was produced by a general leakage of

materials from the organisms. Determination of the level of these acids, indicated that 2-oxoglutarate greatly predominated, showing that the rate of production was constant over the entire range of growth rates from 0.2 to 0.7 $hr.^{-1}$, and decreased at dilution rates lower than 0.2 $hr.^{-1}$. A similar result was obtained from the mixture of substances causing the UV-absorbtion. The similarity between the two suggests that this type of production is not confined to the oxoacids but may be true for other overflow intermediates. It would appear also that the production of all these compounds may have a common mechanism with the production of storage products.

Lipid granules

Another cellular component occurring in the lipid granules of B. megaterium, and generally answering many of the properties of a carbon and energy storage compound, is a homopolyester of β-hydroxybutyric acid. Lemoigne and his co-workers at the Pasteur Institute, in a series of studies from 1923 to 1944, were the first to discover this polyester and to show that it was the major component of the lipid inclusion granules of Bacillus species. These granules had often been observed by the nineteenth century

bacteriologists as refractile bodies in the cytoplasm which they could not stain with the usual dyes. However, it was not until the introduction of Sudan Black B by Hartmann in 1940 that bacterial lipids were studied in earnest. The modification of his method by Burdon et al. (1941) and Burdon (1946) superceded all other methods for staining lipid granules. About this time Lemoigne, Delaporte and Croson (1944), concluded that there was a direct relationship between the amount of PHB which could be extracted and the refractile material exhibited microscopically. Lemoigne improved his original extraction procedure by the use of chloroform to extract the polymer and thus obtained a relatively pure material to carry out a chemical characterisation of the polymer. Subsequent studies by Képès and Péaud-Lenoël (1952) and Williamson and Wilkinson (1958) suggest that it has a polylactide structure, the residue being of various lengths, 60 units on average, depending on the age, source of material, and type of extraction procedure followed. Thus the following structure was suggested for poly-β-hydroxylutyrates:-



Further evidence that the PHB is closely associated with these lipid granules has been provided by Weibull (1953 a & b). In the course of experiments using lysozyme to prepare protoplasts of B. megaterium, he submitted cytoplasmic membranes containing lipid granules to differential centrifugation. Achieving separation of the two he attempted to characterise the lipid inclusions, noting that they stained with Sudan Black B, and were easily soluble in chloroform or warm alkali. Williamson and Wilkinson (1958) studying these experiments in greater detail, noted loss of sudanophilia of the granules subsequent to centrifugation and also by extraction with alkaline hypochlorite, but confirmed that they were directlycomposed of PHB. The tendency of the granules to lose their sudanophilic properties emphasises the caution which must be employed in the interpretation of the cytochemical data obtained from staining procedures. It would appear that the active component reacting with Sudan Black B is not the PHB, but some lipid component on the outside of the granule. Thus if PHB is not stained by Sudan Black B, its presence cannot be conclusively proved by positive staining alone. Therefore the comprehensive tables formed by Burdon (1946) and Williamson (1958) should be viewed with care. A

comprehensive list of specific organisms known to contain PHB has been drawn up by Forsyth, Hayward and Roberts (1958).

Considerable evidence has now been accumulated to show that PHB is best formed in media where there is an excess of the carbon source present and this need not necessarily be carbohydrate as for polysaccharide production (Macrae and Wilkinson, 1958b, Sierra and Gibbons, 1962a & b). The former authors studied the level of PHB in stationary phase cultures of B. megaterium under conditions of glucose deficiency, and excess, i.e. nitrogen limitation. In cells grown under conditions of glucose deficiency the PHB value was low, whereas a high value was obtained under conditions of excess glucose. In the nitrogen-deficient PHB-rich cells, autolysis was delayed. To confirm the lower rate of autolysis, Macrae and Wilkinson compared washed suspensions of cells of B. megaterium rich and poor in PHB. The fall in nitrogen content of washed cells was taken as a measure of autolysis although this might easily have been RNA or protein breakdown. The rate was found to be twice as high in cells poor in polymer as in polymer-rich cells. Manometric studies confirmed

that the endogenous respiration was dependent on the amount of PHB in the cells, previous experiments showing that polysaccharide was not utilised. Macrae and Wilkinson (1958b) studying the anaerobic breakdown of PHB in washed suspensions of PHB-rich cells, noted accumulation of β-hydroxybutyric acid and acetoacetic acid. It was in 1923 that Lemoigne first detected β-hydroxybutyric acid in cells of a Bacillus species. However, depolymerisation occurred most rapidly in aerobic suspensions where most of the depolymerisation products were accounted for by carbon dioxide production. Macrae and Wilkinson (1958b) also demonstrated that in presence of a suitable substrate PHB could be synthesised by washed suspensions of cells, just as in the absence of any substrate, breakdown occurred. Substrates such as glucose, pyruvate, and β-hydroxybutyrate, especially in the presence of acetate, gave maximal synthesis. It should be remembered that for synthesis experiments the cells used were always grown on a defined glucose salts plus 0.1% casamino acids medium with glucose as limiting growth factor. They were harvested approximately 1 or 2 hr. after growth had ceased, from a continuously aerated flask where oxygen was possibly the limiting factor in determining the growth rate. The failure to induce PHB synthesis with many other theoretically suitable substrates is

most probably because they were not permeable to the cell. As pointed out by Wilkinson (1959) the fact that maximum polymer synthesis occurred at pH 7 argues against polymerisation being simply a neutralisation mechanism to dispose of acid end products. The experiments on B. megaterium had demonstrated PHB synthesis, during growth, and in washed cells on suitable substrates; and also depolymerisation and oxidation in the absence of exogenous substrate.

A similar series of results has been obtained by Sierra and Gibbons (1962a & b) with the moderate obligate halophile, Micrococcus halodenitrificans. The medium contained 0.5% amino acids and 1% glycerol to give PHB-rich cells and 1% sodium citrate to give PHB-poor cells. Up to 50% of the dry weight of this coccus could be formed of PHB. In the absence of exogenous substrate washed cell suspensions, rich in PHB, degraded their polymer at a rate consistant with manometric studies. In the polymer-deficient cells no appreciable amount of ammonia was liberated, nor was there any change in carbohydrate, total fat, or viable count for 3 hr.; after this, as the PHB level dropped to 5 % the endogenous metabolism and viability dropped quickly. Using PHB-rich cells the endogenous meta-

bolism and the number of viable cells remained constant for 96 hr. during which time the PHB content dropped to 5%, the endogenous respiration and viability then decreased rapidly.

Poly-B-hydroxybutyrate synthesis has been achieved by using particulate fractions of cell-free extracts of B. megaterium and R. rubrum by Merrick and Doudoroff (1961). The cells of B. megaterium were grown by the method of Macrae and Wilkinson (1958b) and cell-free extracts and polymer particles prepared in a manner similar to that of Weibull (1953). Incubation of the particulate fraction, with or without the supernatant soluble enzymes, plus the addition of radioactive labelled /3 -hydroxybutyryl CoA led to rapid (20 min.) incorporation of 80% of the label into the polymer. Controls containing labelled /3 -hydroxybutyrate and CoA showed no incorporation. Similar extracts of R. rubrum also incorporated the label into the polymer very rapidly (2 min.). As in B. megaterium the activity is largely associated with the particulate fraction. The kinetics of incorporation are however, complicated by the presence of an active depolymerase which is also associated with the particles. The native polymer, resuspended in either the supernatant fraction, or buffer, can undergo as much as 80% hydrolysis (to /3 -hydroxybutyric acid) in 30min.

at 30°.

No direct activation of β-hydroxybutyric acid with ATP and CoA could be demonstrated in cell-free extracts of R. rubrum. A small incorporation of ^{14}C in the polymer was observed however, when crude extracts were incubated with labelled acetate, ATP, CoA and reduced DPN. Two soluble enzymes involved in polymer metabolism were found in the soluble fraction of R. rubrum extracts prepared from cells that had depleted their polymer stores. The hydrolytic system catalyses digestion of boiled polymer particles of R. rubrum and native polymer particles of B. megaterium, but does not attack purified polymer. A specific DPN-linked D(-)β-hydroxybutyric acid dehydrogenase was also present in such extracts.

R. rubrum is a photosynthetic organism and, quite unlike B. megaterium, it photoassimilates acetate, butyrate and β-hydroxybutyrate into PHB. The fate of assimilated, labelled PHB was studied with illuminated washed cells by Stanier et al. (1959) in the presence of ammonium chloride in an atmosphere of nitrogen and carbon dioxide (95 : 5 V/V). More than 90 per cent of the polymer was utilised, much of the ^{14}C being redistributed into other cellular components. In the presence of carbon dioxide and

the absence of a nitrogen source, net synthesis of carbohydrate occurred at the expense of PHB, but in the presence of a nitrogen source and absence of carbon dioxide, little loss of polymer was observed. Thus Stanier, Doudoroff and their co-workers at Berkeley have clearly demonstrated that PHB serves as a store of both carbon and reducing power in R. rubrum. It most probably functions as an energy reserve as well, but no formal account has yet described the oxidative breakdown.

In other organisms, PHB probably does not act as a signiffcant carbon source. Other experiments by Doudoroff and Stanier (1959) using Pseudomonas saccharophila containing labelled PHB, showed that although the polymer acted as a substrate for endogenous respiration, overall transfer to other constitutents did not occur. Similarly, Macrae and Wilkinson (1958b) were unable to show any increase in total nitrogen content when a washed suspension of polymer rich B. megaterium was placed in absence of an external carbon source, but in presence of ammonium ions. The evidence thus seems to show that although PHB may act as a reserve of carbon, energy and reducing power in photosynthetic organisms like R. rubrum, and also chemosynthetic organisms of Hydrogenomonas species which can

also store PHB (Schlegel, Gottshalk and Von Bartha, 1961), will more commonly function as an endogenous energy source, preventing autolysis and death in carbon deficient media. A distinct difference between R. rubrum, and B. megaterium and Ps. saccharophila, was that the relative rates of polymerisation and depolymerisation; in R. rubrum extracts were much more active. It may be that the slow depolymerisation is a means of preserving the stored materials for as long as possible.

How the depolymerised β-hydroxybutyric acid is metabolised is unknown, but Co A fragments may be involved. B. megaterium does not possess a glyoxalate pathway and certainly cannot multiply on acetate alone, but the experiments of Hanson et al. (1963a) show that the organism is capable of oxidising acetate. It would be interesting to compare rates of PHB depolymerisation with the possession of a glyoxalate pathway.

Polyphosphate

The inorganic polyphosphate (volutin) content of microbial cells varies within wide limits, depending upon the organism and the nutritional conditions. In general, accumulation of polyphosphate can be associated with inhibit-

ion of growth provided a source of metabolic energy is still available. Such conditions have been described during starvation of specific nutrients such as sulphur (Smith, Wilkinson and Duguid, 1954) or essential growth factors in auxotrophic mutants (Harold, 1960). In many microbial species, rapid and extensive accumulation of polyphosphate may be induced by adding phosphate to a culture previously deprived of this nutrient (Smith et al. 1954, Harold, 1962).

Recent studies have partly clarified the pathway of polyphosphate biosynthesis. A polyphosphate kinase, occurring in many microorganisms, catalyses the transfer of a terminal phosphate group of ATP to polyphosphate (Kornberg, Kornberg and Simms, 1956). The reaction has been shown to be reversible (Kornberg, 1957), and could thus serve to regenerate ATP from ADP and polyphosphate, suggesting a possible function in microbial energy metabolism. Harold and Sylvan (1963) have shown that regulation of polyphosphate is controlled by the level of oxidised glutathione in cells of Aerobacter aerogenes. These authors also demonstrated that suppression of polyphosphate accumulation in non-growing cells of A. aerogenes required the simultaneous presence of a high exogenous

phosphate concentration and a high intracellular glutathione level. Harold (1963) has also shown that the absence of polyphosphate during growth was due to competition with nucleic acid synthesis for intracellular phosphorus. Polyphosphate accumulated only after nucleic acid synthesis had ceased. Resumption of nucleic acid synthesis (with or without concurrent cell growth) induced rapid degeneration of the polyphosphate with transfer of phosphorus to RNA, apparently via inorganic phosphate.

Conclusion

From the foregoing review of growth and storage materials, it can be appreciated that the latter have a widespread occurrence in microorganisms and in many cases their function as true storage compounds has been demonstrated. However, most investigations into the conditions of their production have only examined nitrogen deficient conditions. Even in nitrogen deficiency, there is a dearth of ancillary information regarding the general quantitative conditions under which storage compounds are produced.

In the following chapters in this thesis it is shown, that by using a chemostat to control the chemical environment, how production, not only of some of the aforementioned storage materials, but also of other cellular

properties and components is affected by different limiting nutrients. To this effect, Bacillus megaterium, an organism known to accumulate PHB, glycogen and volutin granules, has been grown continuously for periods of up to ten weeks in two similar types of chemostat which are described in detail in the following chapters. The effect of carbon and mineral limitations, and the use of the apparatus to control the growth rate, has been used to examine the levels of these storage materials, the morphology and yield of cell material per unit of limiting nutrient, and also the cell content of RNA and DNA. Certain genetical changes have been examined for in the population. The rate of metabolism of glucose and the production of certain extracellular products was also determined. Taken together the data assembled help in an understanding of the complex problems in microbial physiology at a molecular as well as a cellular level.

IV

APPARATUS

CONTINUOUS CULTURE APPARATUS

The mass propagation of microorganisms is the most direct application of continuous culture methods and is especially suited to single stage operation. Probably the only significant industrial continuous cell processes today are those involved in yeast manufacture, including alcohol production and the disposal of waste sulphite liquors. These processes (barring alcohol production) and many other industrial fermentations involve the use of deep culture vessels equipped to give good aeration and agitation. One such type, called the Waldhof system, was developed and used extensively during the Second World War in Germany for the production of food yeast. The Waldhof fermentor is unique in that both air and agitation are supplied in a single hollow bladed impeller which is positioned beneath a central draught tube; foam circulates down this tube and out into the turbulent emulsion by means of the centrifugal action of the impeller. A high power input and a low operating capacity of the total fermentor volume are two limitations; effective foam dispersion and very high oxygen solution rates are two big advantages in its favour.

All manner of laboratory continuous culture vessels

have been described from approximately 50 ml. in volume (Schaechter et al. 1958) to 10 l. (Pirt and Callow, 1961) A continuous culture device, if large enough, will usually produce a gross excess of cellular material, but there is a vessel size above which the provision of fresh medium can become an embarrassment. The minimum capacity is defined largely by the ability to fit such features as a successful agitation system, pH electrodes and other probes. In designing a vessel to suit all requirements the following three examples in Table 1 help to decide the size of vessel and the ranges over which the pump will be required to operate.

Table 1

Doubling Time (Hrs.)	Equivalent Dilution Rate ($Hr.^{-1}$)	Rate of Medium Flow in a Chernostat per l. of vessel capacity.	
		mls./hr.	l/24 hr.
0.33	2.07	2,070	50
3.00	0.23	230	5.5
14.00	0.05	49.5	1.2

If many differing types of organism are to be grown, each having individual requirements, then as many variable design factors as possible must be included.

Many apparatus have been described in greater or

less detail, but it is indeed unique that the Microbiological Research Establishment, at Porton Down, have provided so much data on continuous culture vessels of laboratory size, and the related equipment for their continuous and automatic running. While the designs bear much resemblence to standard industrial types, they contain many refinements, necessarily developed to withstand continuous unbroken periods of use running into hundreds and often thousands of hours. Thus Elsworth et al. (1956) described, in exhaustive detail, a 2 l. continuous culture apparatus of the chemostat type. Dimensions and materials of construction, including the seal on the shaft, were described, its performance in sulphite oxidation rates and mixing efficiency, and also systems for controlling temperature, foaming and the measurement of oxygen and carbon dioxide in the effluent air. Detailed accounts of the harvesting system, how to sterilize the apparatus and air supply, charging fresh medium, inoculation and sampling, were also included. A separate account (Pirt and Callow, 1956) described the automatic pH control system. Thus all relevant techniques were discussed with relation to one vessel. Further improvements to the stirrer gland were described later (Elsworth et al. 1958).

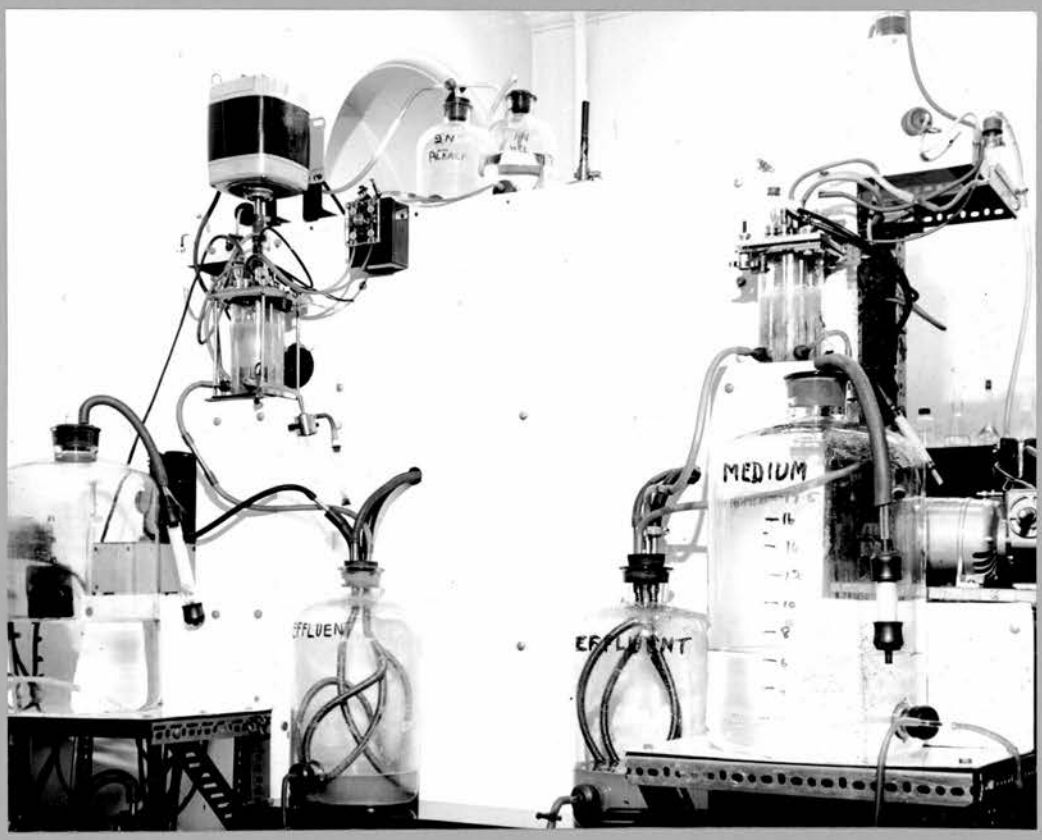


Fig. 1: Frame supporting the two chemostats.

GENERAL DESCRIPTION

The two apparatus described in this thesis are both of the single stage chemostat type. One is of approximately 500 ml. capacity, fitted with a conventional gland on the stirrer shaft, and is a miniature of the Waldhof fermentor. The author owes most of the design of the vessel top, and agitation system to Dr. D. Herbert. The second apparatus is of approximately 400 ml. capacity, with a similar Waldhof baffling tube, and is fitted with a magnetic stirrer. Sulphite oxidation studies have proved that both vessels are capable of high oxygen transfer rates.

Both vessels are mounted at opposite ends of a Dexion frame, 5 ft. 6 ins. long and 5 ft. 6 ins. high, faced in white perspex (Fig 1.). Common services are shared on this frame and convenient shelves are placed for holding medium, effluent, acid, alkali and antifoam bottles and medium pumps. This frame also mounts the air filters and refrigerator and brine bath. A second Dexion frame (Fig 2.) 6 ft. high and 3 ft. 6 ins. long, mounts the electrical supply (Fig. 3.), the electronic controlling devices and the air supply. At one side are mounted the pH meter, autotitrator controller and recorder, and the other is filled with an aluminium sheet which supports temperature con-

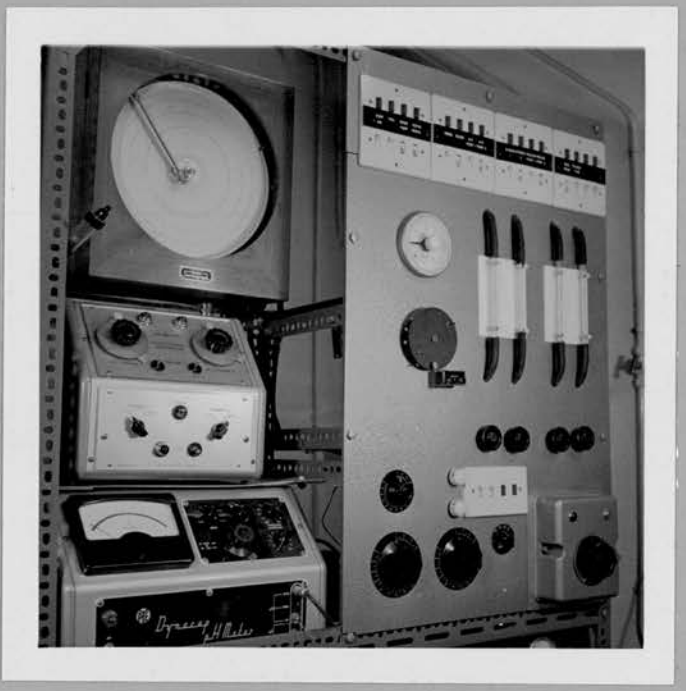


Fig. 2: Control frame supporting electrical and compressed air services.

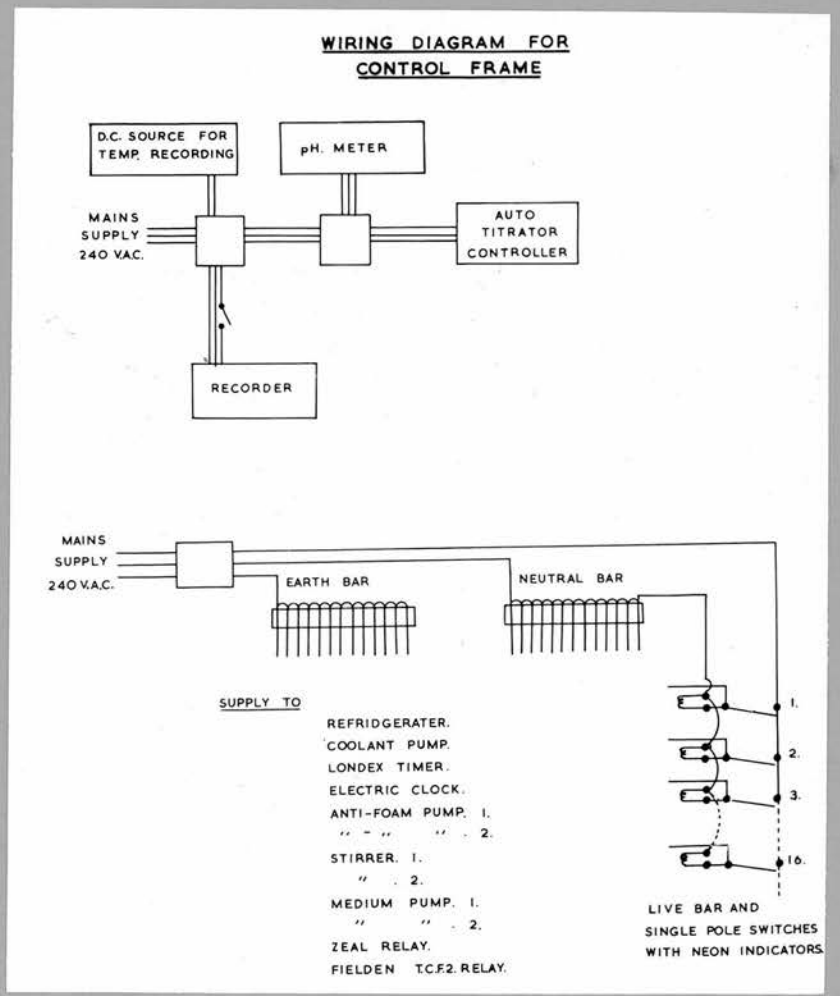
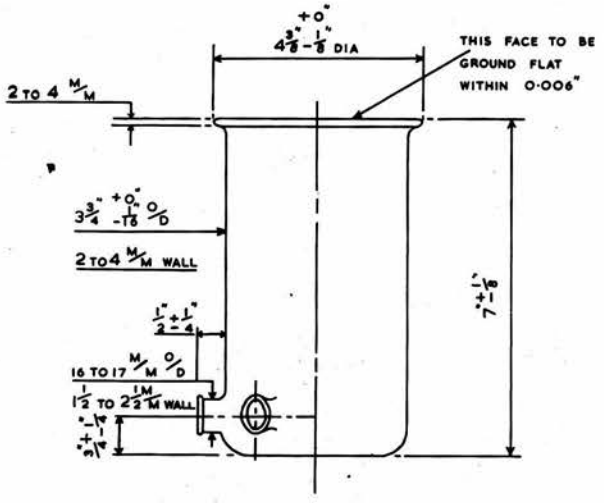


Fig. 3.

FERMENTOR VESSEL



PLAN

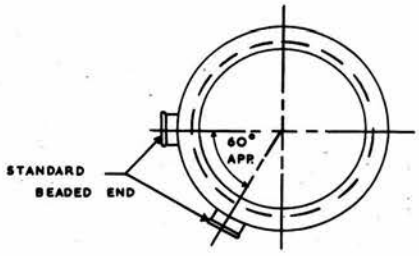


Fig. 4.

trollers, air flow meters, the automatic antifoam addition devices, and at the top the electrical switches.

FERMENTOR 1

The fermentor vessel is made of glass, (Fig. 4.) has a flat bottom, and two ports near the bottom on the side. The flange at the top of the vessel is ground flat and a neoprene washer effects a seal between it and the stainless steel top plate. The base of the vessel sits on another stainless steel plate protected by another washer of neoprene. Four $\frac{1}{4}$ in. rods, threaded at the top, are brazed into each corner of the baseplate, and fit into respective holes in the top plate. Thus the vessel is held between the top and bottom plates between two neoprene washers and is bolted by four wing nuts. The top plate and one of the eight ports, are shown in Fig. 5. and 6. respectively. The following probes or services are entered in one of the ports;- pH electrode, a resistance thermometer for temperature control, a resistance thermometer for temperature recording, an inoculation port, a 'cold finger', acid alkali and antifoam inlet points, a medium feed, and a spare. The central position is filled by the stirrer gland (Figs. 7, 8,). The two glass ports at the

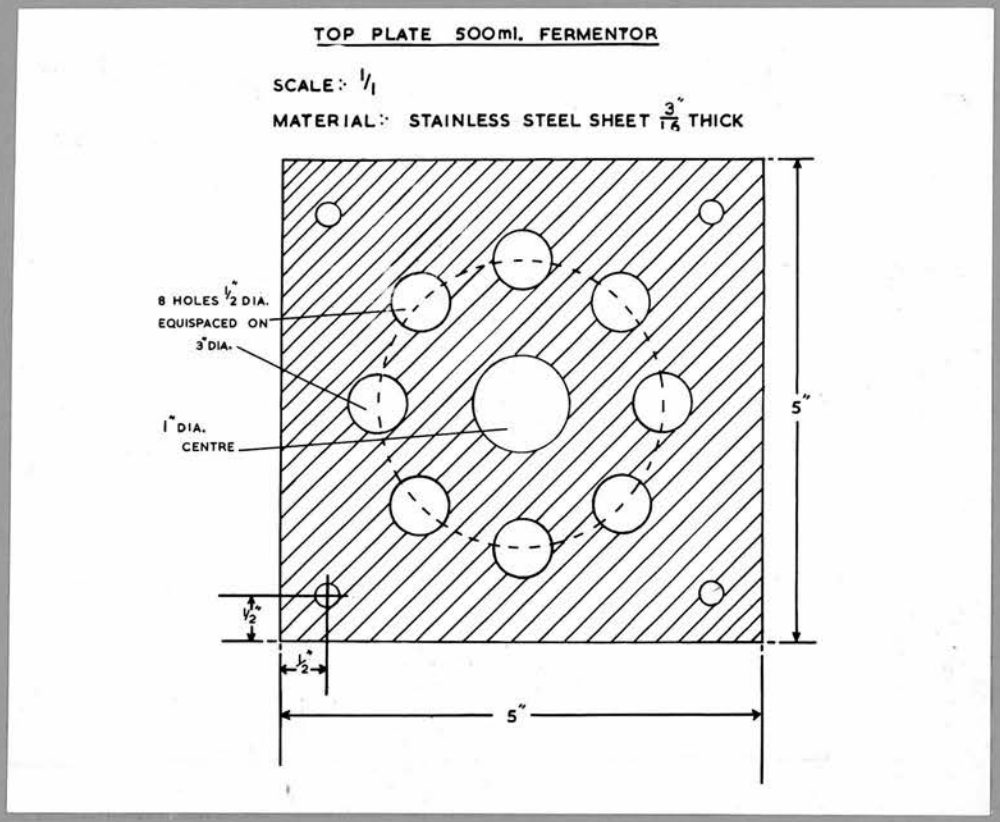

TOP PLATE 500ml. FERMENTOR
SCALE :- 1/1
MATERIAL :- STAINLESS STEEL SHEET 3/16" THICK
8 HOLES 1/2" DIA. EQUISPACED ON 3" DIA.
1" DIA. CENTRE
5"
1/2"
1/2"
5"

Fig. 5.

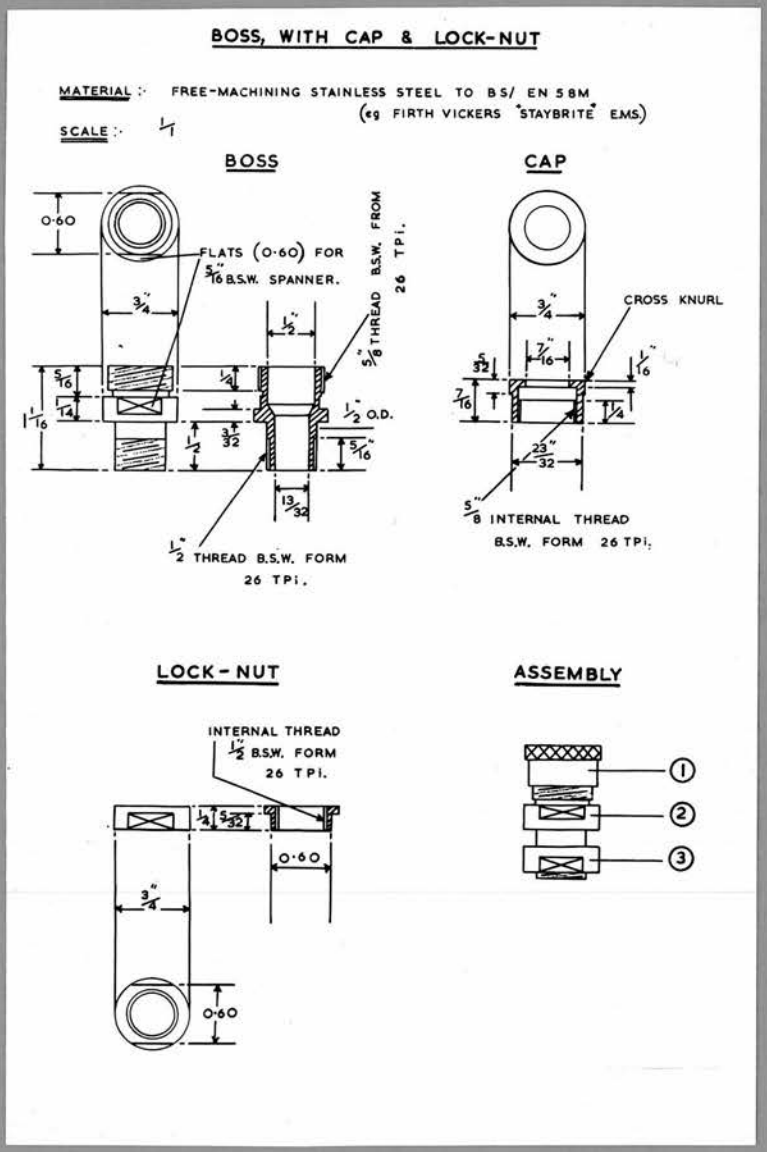

BOSS, WITH CAP & LOCK-NUT
MATERIAL :- FREE-MACHINING STAINLESS STEEL TO BS/ EN 58M
(eg FIRTH VICKERS "STAYBRITE" EMS)
SCALE :- 1/1
BOSS
CAP
0·60
FLATS (0·60) FOR 5/16 B.S.W. SPANNER.
5/8" THREAD B.S.W. FROM 26 TPi.
3/4"
1/2"
5/16
1/4
1 1/16
1/2
3/32
1/2" O.D.
5/16"
13/32
1/2" THREAD B.S.W. FORM 26 TPi.
CROSS KNURL
7/16"
5/32
1/16"
7/16
1/4
23/32"
5/8" INTERNAL THREAD B.S.W. FORM 26 TPi.
LOCK-NUT
ASSEMBLY
INTERNAL THREAD 1/2" B.S.W. FORM 26 TPi.
5/32
0·60
3/4"
0·60
1
2
3

Fig. 6.

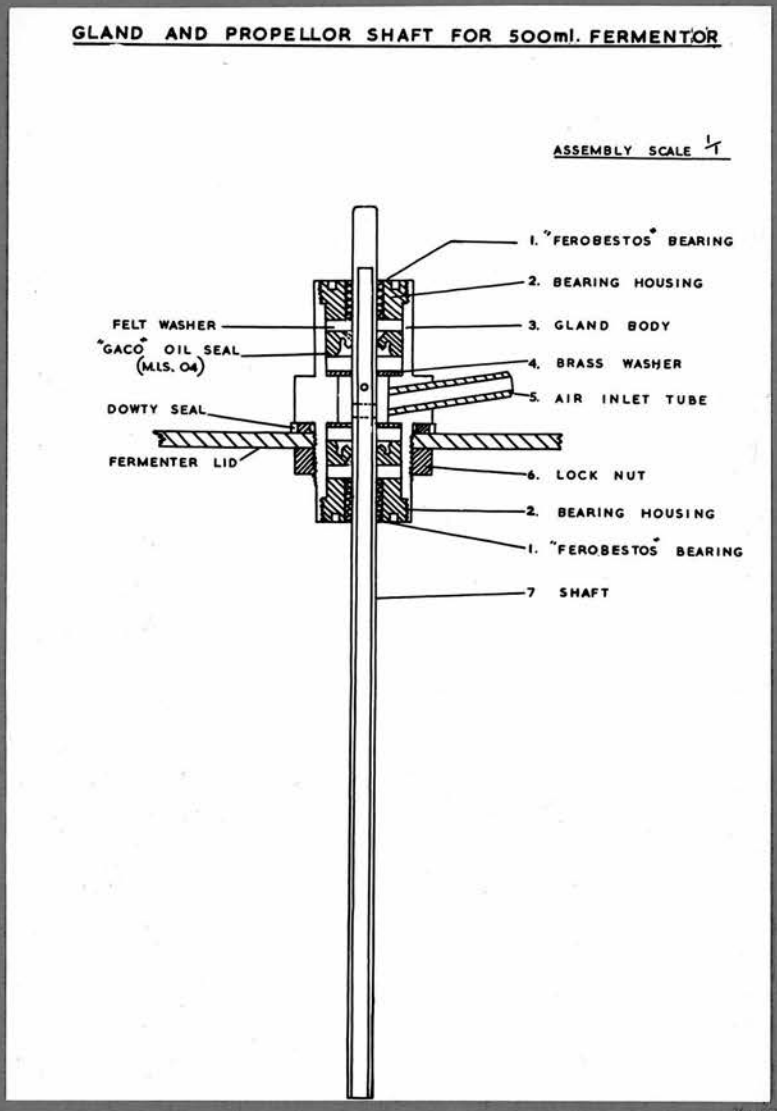

GLAND AND PROPELLOR SHAFT FOR 500ml. FERMENTOR
ASSEMBLY SCALE 1/1
1. "FEROBESTOS" BEARING
2. BEARING HOUSING
3. GLAND BODY
4. BRASS WASHER
5. AIR INLET TUBE
6. LOCK NUT
2. BEARING HOUSING
1. "FEROBESTOS" BEARING
7 SHAFT
FELT WASHER
"GACO" OIL SEAL (M.I.S. 04)
DOWTY SEAL
FERMENTER LID

Fig. 7.

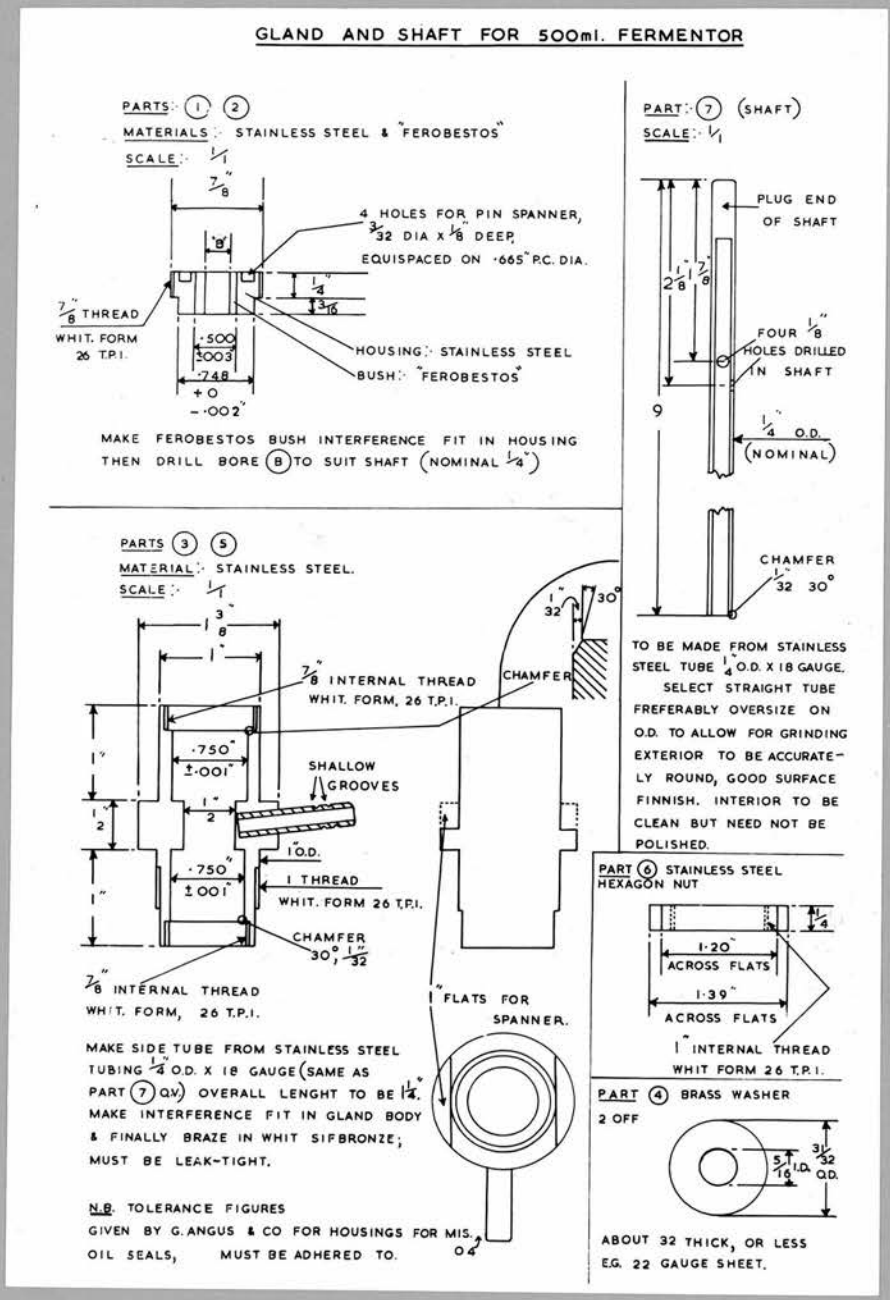

GLAND AND SHAFT FOR 500ml. FERMENTOR
PARTS: (1) (2)
MATERIALS: STAINLESS STEEL & "FEROBESTOS"
SCALE: 1/1
7/8"
4 HOLES FOR PIN SPANNER, 3/32 DIA X 1/8" DEEP, EQUISPACED ON ·665" P.C. DIA.
1/4"
3/16
7/8" THREAD WHIT. FORM 26 T.P.I.
·500 +·003
·748 +0 −·002"
HOUSING: STAINLESS STEEL
BUSH: "FEROBESTOS"
MAKE FEROBESTOS BUSH INTERFERENCE FIT IN HOUSING THEN DRILL BORE (8) TO SUIT SHAFT (NOMINAL 1/4")
PART: (7) (SHAFT)
SCALE: 1/1
PLUG END OF SHAFT
2 1/8"
1 7/8"
FOUR 1/8" HOLES DRILLED IN SHAFT
9
1/4" O.D. (NOMINAL)
CHAMFER 1/32" 30°
TO BE MADE FROM STAINLESS STEEL TUBE 1/4" O.D. X 18 GAUGE.
SELECT STRAIGHT TUBE FREFERABLY OVERSIZE ON O.D. TO ALLOW FOR GRINDING EXTERIOR TO BE ACCURATELY ROUND, GOOD SURFACE FINNISH. INTERIOR TO BE CLEAN BUT NEED NOT BE POLISHED.
PARTS (3) (5)
MATERIAL: STAINLESS STEEL.
SCALE: 1/1
1 3/8"
1"
7/8" INTERNAL THREAD WHIT. FORM, 26 T.P.I.
CHAMFER
1/32" 30°
1"
·750" ±·001"
SHALLOW GROOVES
1/2"
1"
1" O.D.
·750" ±·001"
1" THREAD WHIT. FORM 26 T.P.I.
CHAMFER 30° 1/32"
7/8" INTERNAL THREAD WHIT. FORM, 26 T.P.I.
1" FLATS FOR SPANNER.
MAKE SIDE TUBE FROM STAINLESS STEEL TUBING 1/4" O.D. X 18 GAUGE (SAME AS PART (7) Q.V.) OVERALL LENGHT TO BE 1 1/4". MAKE INTERFERENCE FIT IN GLAND BODY & FINALLY BRAZE IN WHIT SIFBRONZE; MUST BE LEAK-TIGHT.
N.B. TOLERANCE FIGURES GIVEN BY G. ANGUS & CO FOR HOUSINGS FOR MIS. OIL SEALS, MUST BE ADHERED TO.
PART (6) STAINLESS STEEL HEXAGON NUT
1/4"
1·20" ACROSS FLATS
1·39" ACROSS FLATS
1" INTERNAL THREAD WHIT FORM 26 T.P.I.
PART (4) BRASS WASHER
2 OFF
5/16" I.D.
31/32" O.D.
ABOUT 32 THICK, OR LESS E.G. 22 GAUGE SHEET.

Fig. 8.

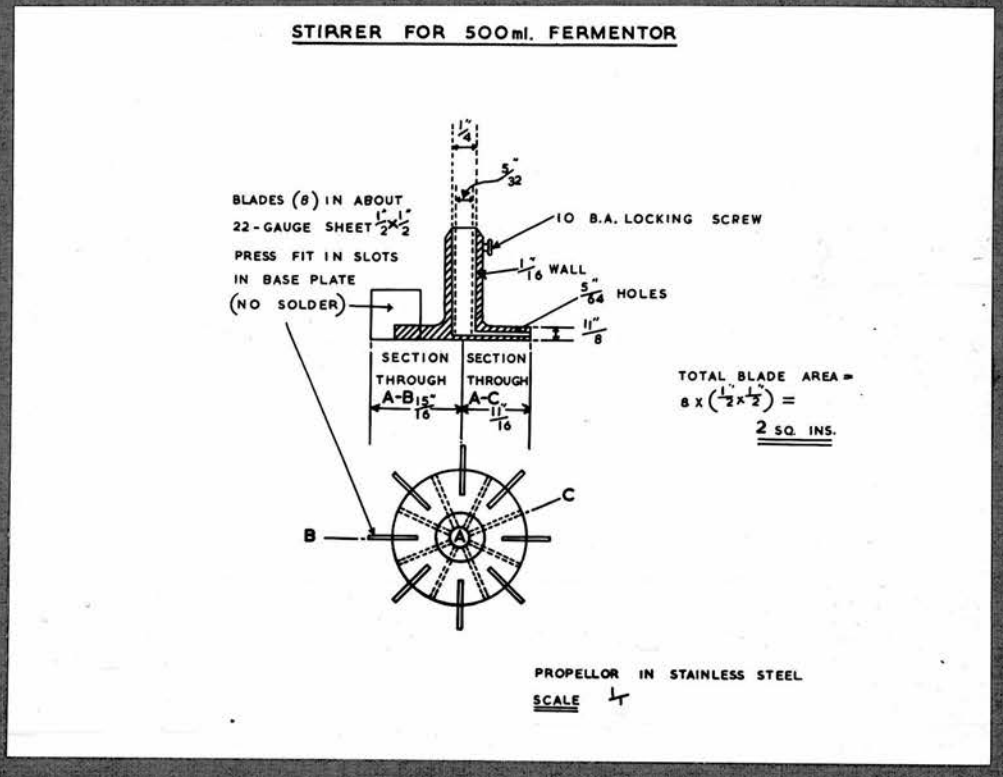

STIRRER FOR 500ml. FERMENTOR
1/4"
5/32"
BLADES (8) IN ABOUT 22-GAUGE SHEET 1/2" x 1/2"
PRESS FIT IN SLOTS IN BASE PLATE (NO SOLDER)
10 B.A. LOCKING SCREW
1/16" WALL
5/64" HOLES
1/8"
SECTION THROUGH A-B 15/16"
SECTION THROUGH A-C 11/16"
TOTAL BLADE AREA = 8 x (1/2" x 1/2") = 2 SQ. INS.
A
B
C
PROPELLOR IN STAINLESS STEEL
SCALE 1/1

Fig. 8a.

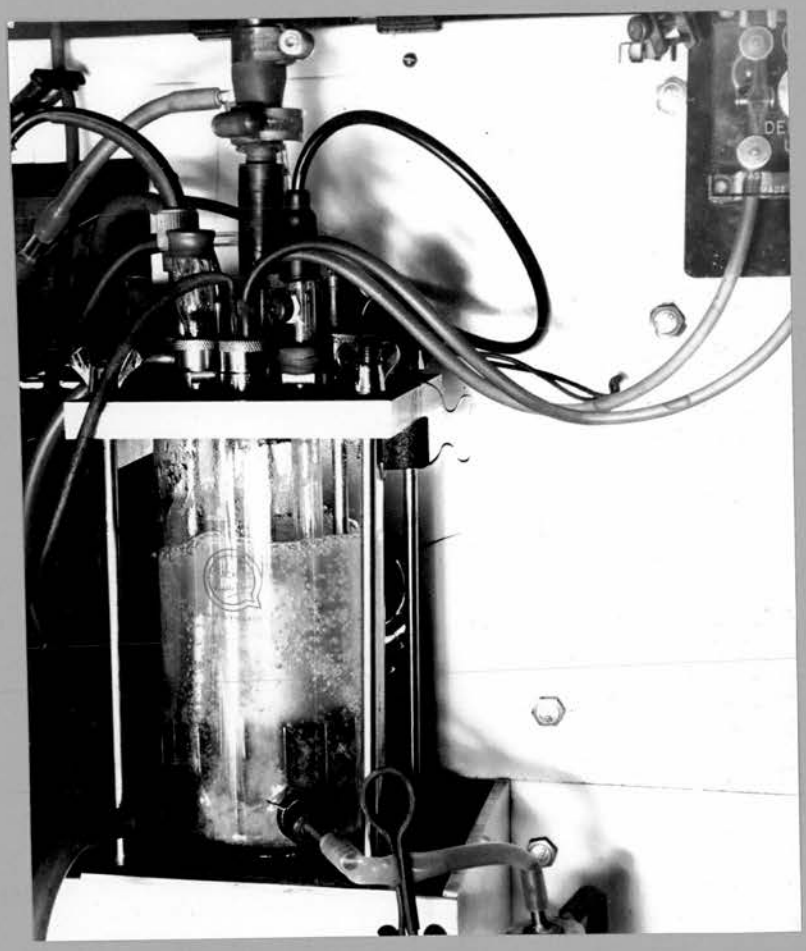


Fig. 9. Fermentor 1.

side of the vessel are used for a sampling point and for an overflow device. The assembly is shown in Fig. 9.

Seals and Joints

Sealing the ground glass top of the vessel and the stainless steel plate is effected by the use of a neoprene ring $3\frac{1}{2}$ ins. I.D. by $4\frac{1}{2}$ ins. by $\frac{1}{8}$ ins. thickness and shore hardness 60 (James Walker & Son Ltd.)*. The shaft seal (Figs. 7, 8) is obtained by using two 'Gaco' MI oil seals (George Angus & Co. Ltd.). The construction of the gland allows sterile air to enter between the two thus giving a positive pressure inside the gland, preventing laboratory infection via the top half and leakage of the culture into the gland via the bottom half. Both have felt washers impregnated with M544 silicone bearing grease (Midland Silicones Ltd.) on either side. The shaft is supported at either end of the gland by a bearing of plastic material called Ferrobestos (Roberts & Co. Ltd.). This material is unaffected by autoclaving, is selflubricating because graphite is incorporated, and wears well if the shaft is round and straight.

The entry ports to the vessel are not welded to the top plate, but are screwed down; the seal is made by use

*An appendix of firms mentioned in the text and the materials they supply is given at the back.

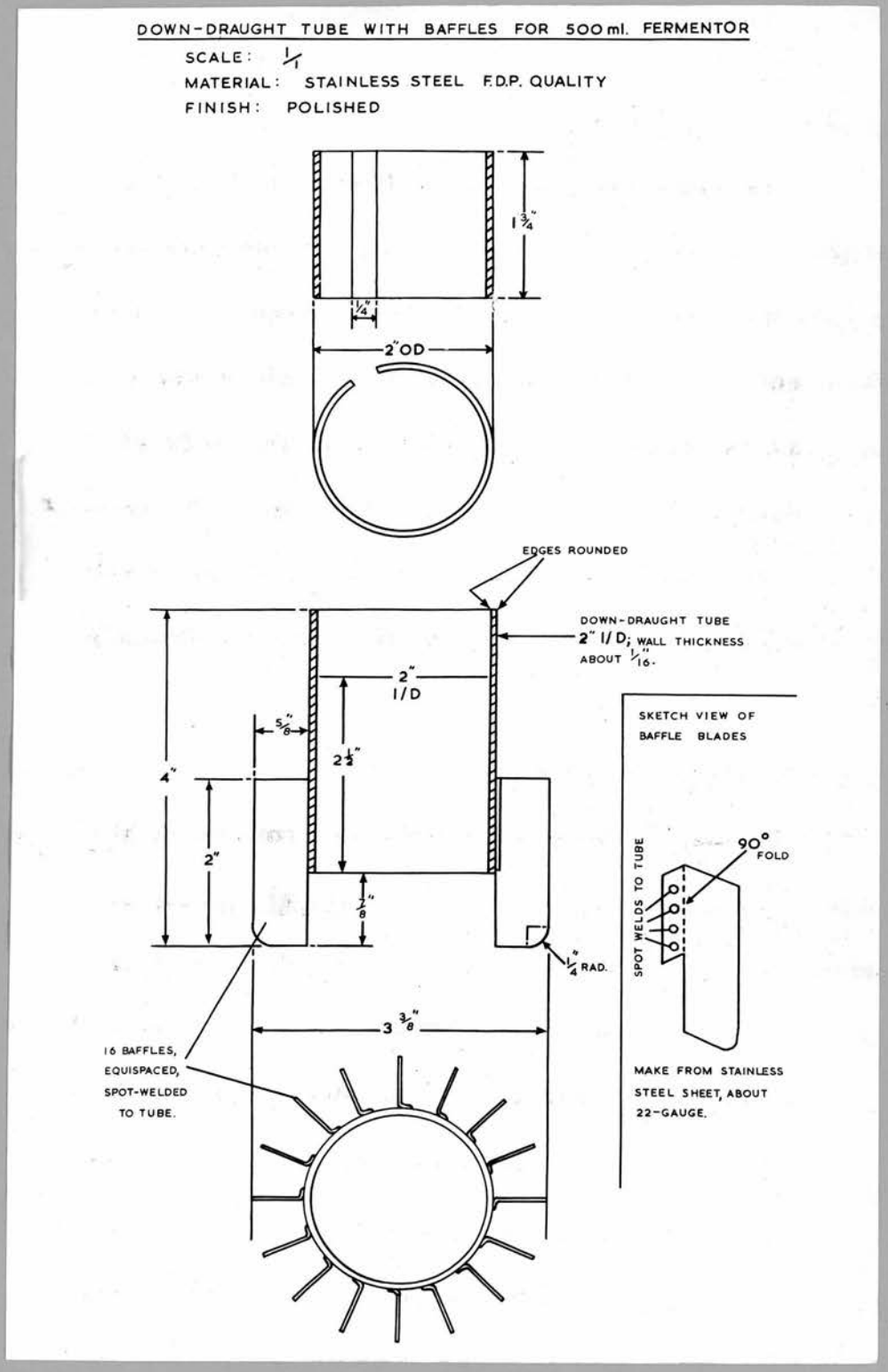

DOWN-DRAUGHT TUBE WITH BAFFLES FOR 500 ml. FERMENTOR
SCALE: 1/1
MATERIAL: STAINLESS STEEL F.D.P. QUALITY
FINISH: POLISHED
1 3/4"
1/4"
2" OD
EDGES ROUNDED
DOWN-DRAUGHT TUBE 2" I/D; WALL THICKNESS ABOUT 1/16".
2" I/D
5/8"
2 1/2"
4"
2"
1/8"
1/4 RAD.
3 3/8"
16 BAFFLES, EQUISPACED, SPOT-WELDED TO TUBE.
SKETCH VIEW OF BAFFLE BLADES
SPOT WELDS TO TUBE
90° FOLD
MAKE FROM STAINLESS STEEL SHEET, ABOUT 22-GAUGE.

Fig. 10.

of a Bonded Seal. (Dowty Seals Ltd.).

Aeration and Agitation

The down draught tube is shown in Fig. 10. The height is set at $\frac{3}{4}$ ins. below the top of the overflow tube to give maximum conditions for foam breaking. Air, which enters two holes in the hollow shaft in the centre of the gland is dispensed through holes in the body of the eight bladed impeller (Fig. 8a). The top of the impeller blades are situated $1/8$ ins. below the cylindrical body of the down draught tube giving amaximum circulation and aeration at this point.

Sulphite Oxidation Rates

Oxygen solution rates were determined by the sulphite oxidation method of Cooper et al. (1944) as described by Elsworth et al. (1957). Figs. 11 and 12 show the range of oxygen solution rates obtained in the apparatus and the conditions necessary to produce them. The highest oxygen solution rate obtained was 510 millimolecules of 0_2/l/hr. A fixed stirring speed of 1440 r.p.m. and an air flow rate of 500 ml/min. were adopted throughout all growth experiments in this vessel.

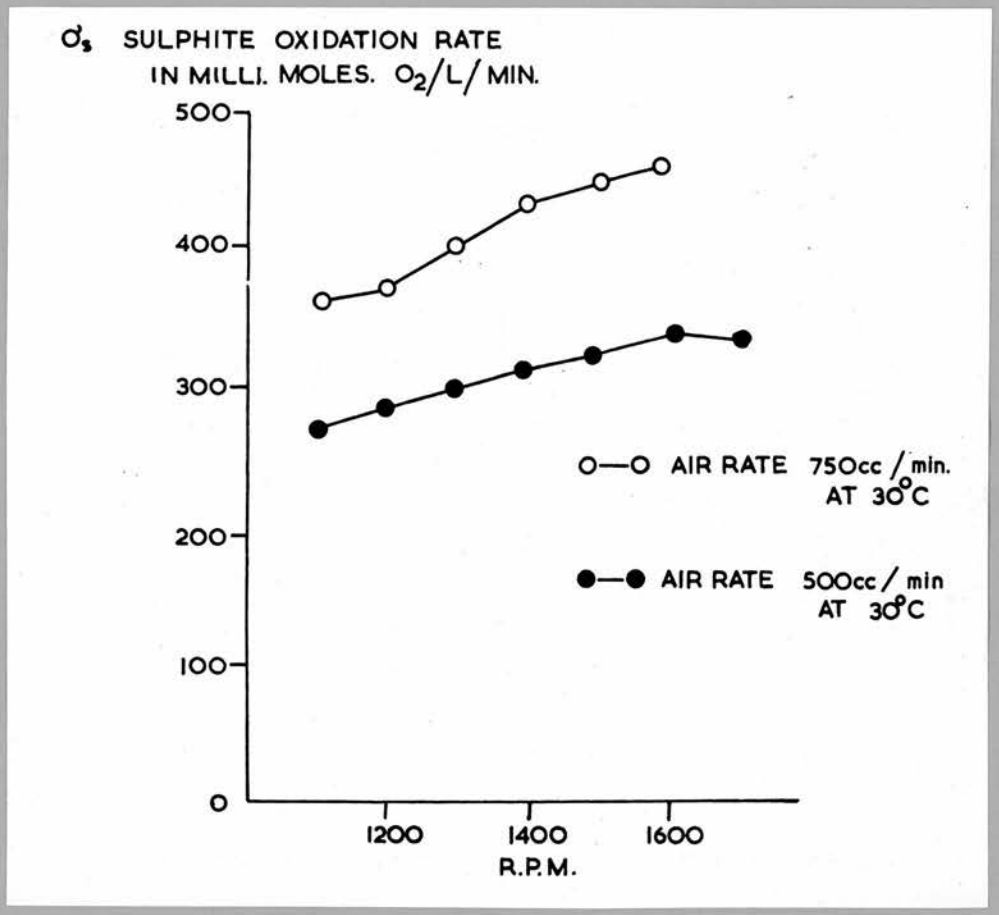


Fig. 11: The relationship between oxygen solution rate (Cu catalysed sulphite oxidation) and the stirrer speed. Volume of solution, 500 ml. in Fermentor 1. The top of the stirrer blades were 1/8 in. below the bottom of the cylinder forming the down-draught tube.

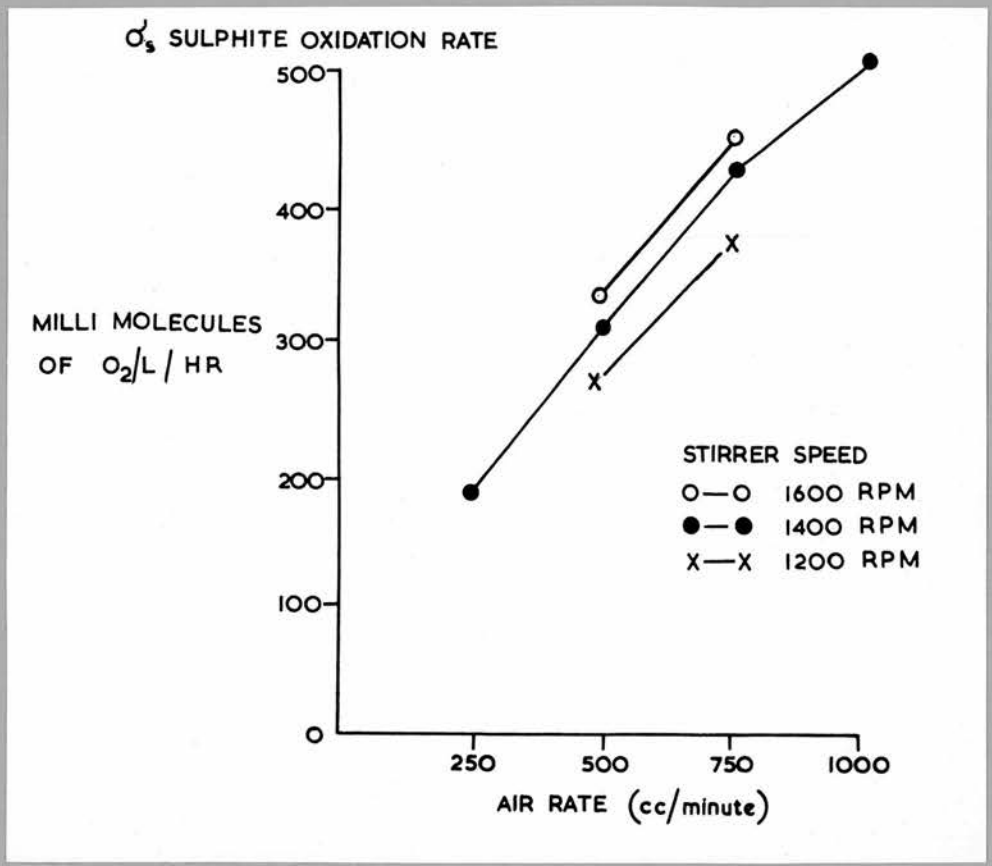


Fig. 12: The relationship between oxygen solution rate (Cu-catalysed sulphite oxidation) and the air flow rate. Volume of solution, 500 ml. in Fermentor 1. The top of the stirrer blades were 1/8 in. below the bottom of the cylinder forming the down-draught tube.

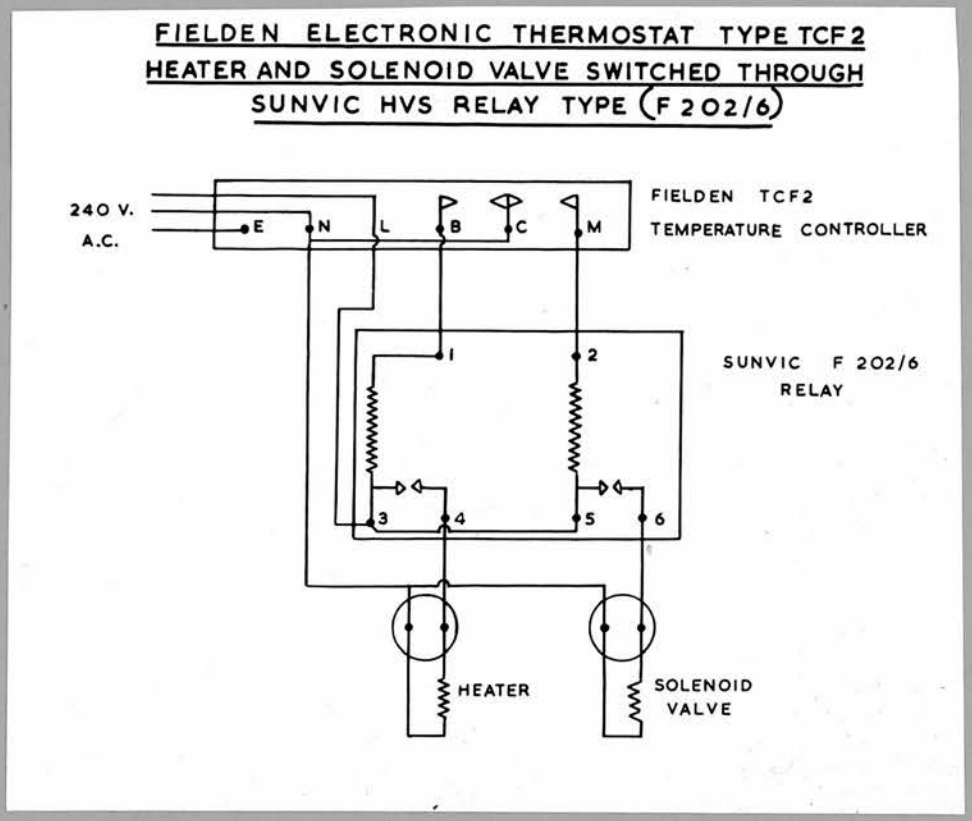

FIELDEN ELECTRONIC THERMOSTAT TYPE TCF2
HEATER AND SOLENOID VALVE SWITCHED THROUGH
SUNVIC HVS RELAY TYPE (F 202/6)
240 V.
A.C.
E
N
L
B
C
M
FIELDEN TCF2
TEMPERATURE CONTROLLER
1
2
SUNVIC F 202/6
RELAY
3
4
5
6
HEATER
SOLENOID
VALVE

Fig. 13.

Temperature Control

The temperature controller was made by Fielden Instruments Ltd. (Model TCF 2) and has a control range 20 - 40°. The circuit is shown in Fig. 13. A rise in temperature causes a 150 watt infrared lamp to be switched off, while simultaneously another pair of contacts closes and supplies a restoring impulse by opening a magnetic valve (Teddington Instruments Ltd.) which passes cold water through the 'cold finger' (Fig. 14). As the temperature falls below the control setting, the cooling water is switched off and the heater is switched on. The monitoring signal is transmitted to the controller by a resistance thermometer (Weston Resistance bulb model S110, Sangamo Weston Ltd.). The system has proved satisfactory for an operating temperature of 30°. For endothermic conditions the accuracy of control is ± 0.1° or even better. It has been noted that at 1440 r.p.m. the stirrer raised 500 ml. of water to 27° from an ambient temperature of 17°.

Temperature Recording

Temperature is recorded in both apparatus by a system independent of the respective temperature controlling mechanisms. The circuit is shown in Fig. 15. An

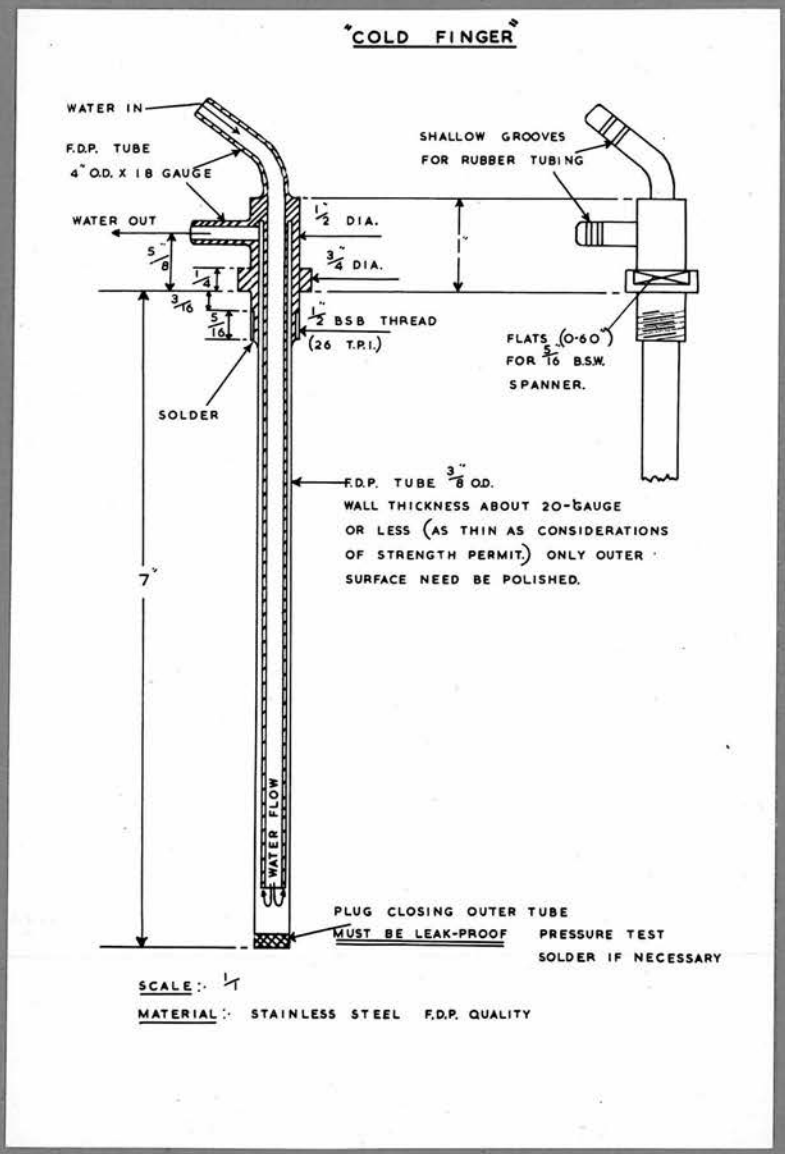

"COLD FINGER"
WATER IN
F.D.P. TUBE
4" O.D. X 18 GAUGE
WATER OUT
SHALLOW GROOVES
FOR RUBBER TUBING
1/2 DIA.
3/4 DIA.
5/8
1/4
3/16
5/16
1/2" BSB THREAD
(26 T.P.I.)
FLATS (0·60")
FOR 5/16 B.S.W.
SPANNER.
SOLDER
F.D.P. TUBE 3/8" O.D.
WALL THICKNESS ABOUT 20-GAUGE
OR LESS (AS THIN AS CONSIDERATIONS
OF STRENGTH PERMIT.) ONLY OUTER
SURFACE NEED BE POLISHED.
7"
WATER FLOW
PLUG CLOSING OUTER TUBE
MUST BE LEAK-PROOF
PRESSURE TEST
SOLDER IF NECESSARY
SCALE :- 1/1
MATERIAL :- STAINLESS STEEL F.D.P. QUALITY

Fig. 14.

8 V DC supply is placed across a Wheatstone Bridge, one arm of which is a Weston resistance bulb of approximately 150 Ohms. Two other arms are fixed at 150 Ohms. and the fourth is a variable resistance purposely offset in order to produce a current of approximately 200 - 300 microamps in the recorder. Only a change in the ambient temperature of the culture round the tip of the resistance bulb causes a variation on the recorder pen. As the 8V DC current comes from a combined transformer and rectifier supplied from the mains, apparent drops in temperature occur at peak loading periods, especially during the winter. The circuit was originally designed to show failure of a heating element, but was sufficiently good to show the oncoming failure of the two amplifier valves in the Fielden TCF 2 thermostat when they gave a temperature variation of $\pm$ 0.3°.

Recorder

A 4 point Fielden Servograph Mark II giving a 24 hr. circular chart was used. The instrument has a 500 microamp full scale deflection and is coupled directly across the temperature indicating Wheatstone Bridge.

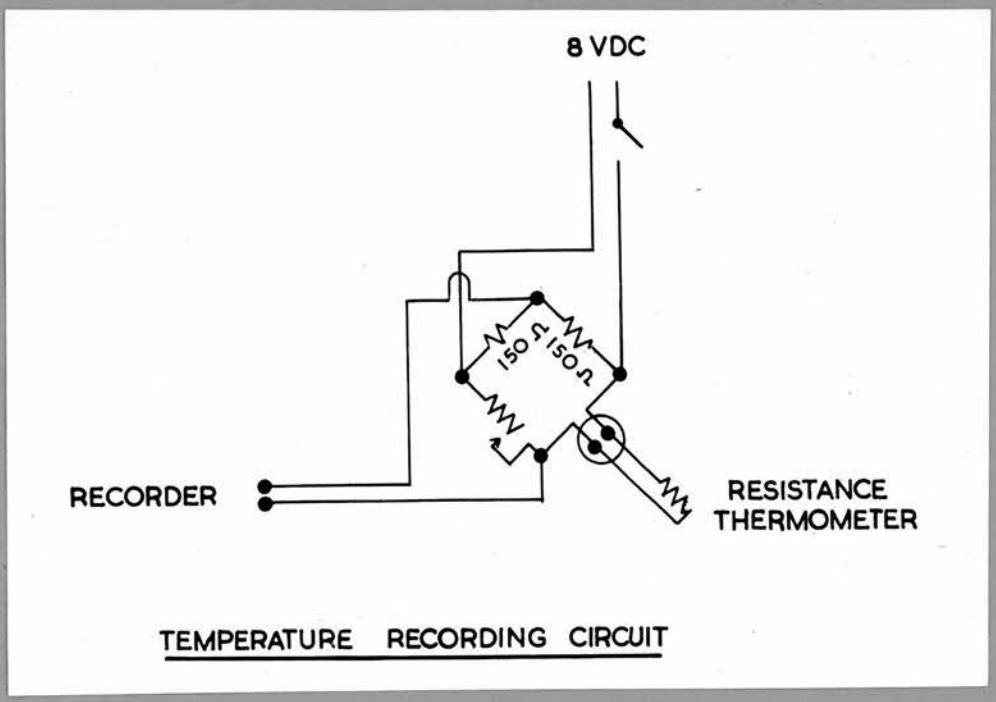

8 VDC
150 Ω
150 Ω
RECORDER
RESISTANCE
THERMOMETER
TEMPERATURE RECORDING CIRCUIT

Fig. 15.

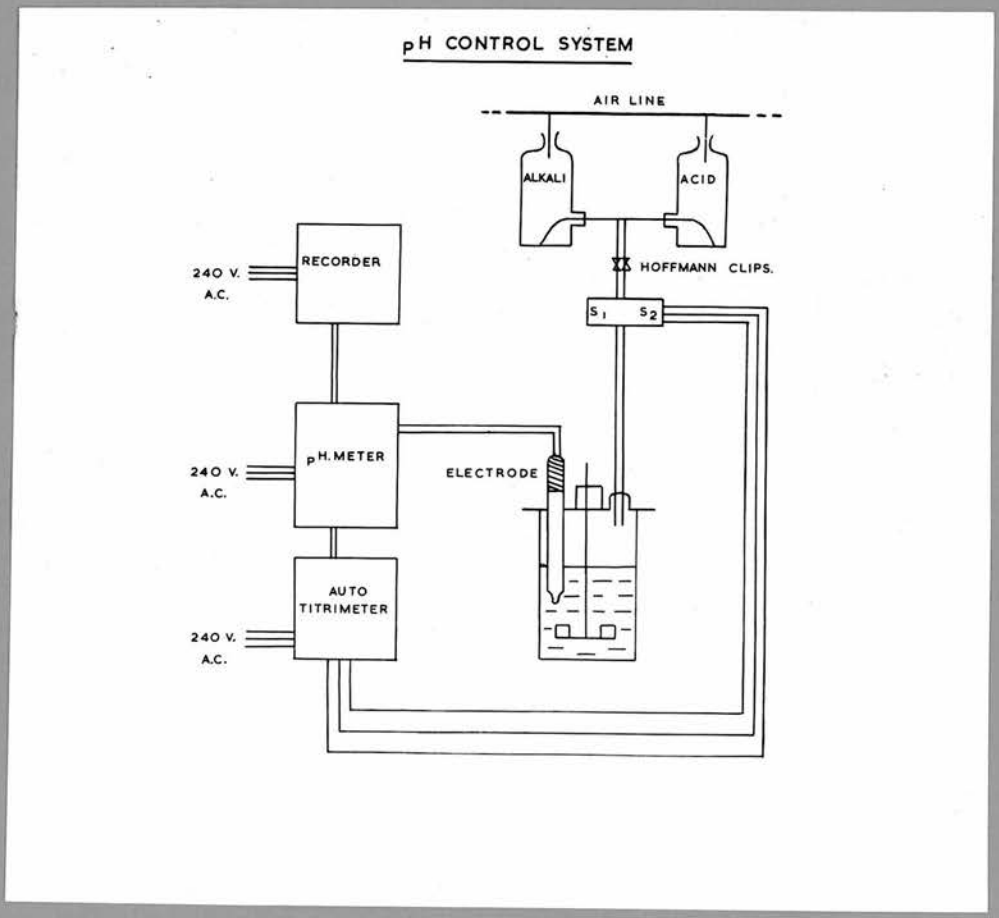

pH CONTROL SYSTEM
AIR LINE
ALKALI
ACID
HOFFMANN CLIPS.
S1
S2
240 V. A.C.
RECORDER
240 V. A.C.
pH. METER
ELECTRODE
240 V. A.C.
AUTO TITRIMETER

Fig. 16.

pH Control System

The assembly is shown schematically in Fig. 16. The pH meter used, covering the range 0 - 14 pH, was a Dynacap (W.G. Pye & Sons Ltd.) having an effective scale length on the expanded ranges of 35 ins. A Pye Autotitrator Controller was used in conjunction with the Dynacap. It contains two independent difference amplifiers, each controlling a relay at a desired value of the input voltage. As a controller this means that it has two channels, which may be used independently, in such a way that one channel may control the addition of alkali and the other acid. Thus, it is particularly suited for holding a culture of growing bacteria at a constant pH. The delivery unit (Pye Catalogue No. 11610) comprises two electrically operated valves mounted side by side. In the closed position, with no current passing through the solenoids, the actuating arms are pulled down by springs and the rollers squeeze the flexible tubes against the front panel so stopping the flow of reagent. When a solenoid is energised, the pressure on the tubing is released and reagent flows through the tubing, the rate being controlled by a Hoffmann Clip.

The pH electrode was an Ingold, Type 405,

(W.G. Pye & Co. Ltd.) which contains both the reference and glass electrodes combined in the one stalk. The electrodes have proved suitable for continuous running over many thousands of hours without failure. The electrode is sterilised chemically, first by immersion to the level of the Suba-seal bung which holds it in place during operation, in 2.5% lysol for 24 hrs; secondly, after a brief wash in sterile water, it is transferred directly into a flask containing 200 ml. of ethylene oxide. Sterile cotton wool is wrapped round the top of the electrode above the bung to prevent infection from aerosols. The ethylene oxide is then boiled off in a fume cupboard over a period of 3 hrs. Finally, the electrode is removed from the flask and inserted into the port on the vessel top. This operation is carried out as suggested by Callow and Pirt (1956) against a stream of sterile air from the inside of the vessel to minimise the change of contamination.

Standardisation of the electrode was carried out approximately once every 4 days by the methods of Callow and Pirt (1956). After the first 100 hrs. it was unusual to have to alter the pH setting. The degree of control of pH by this system is dependent on the rate of

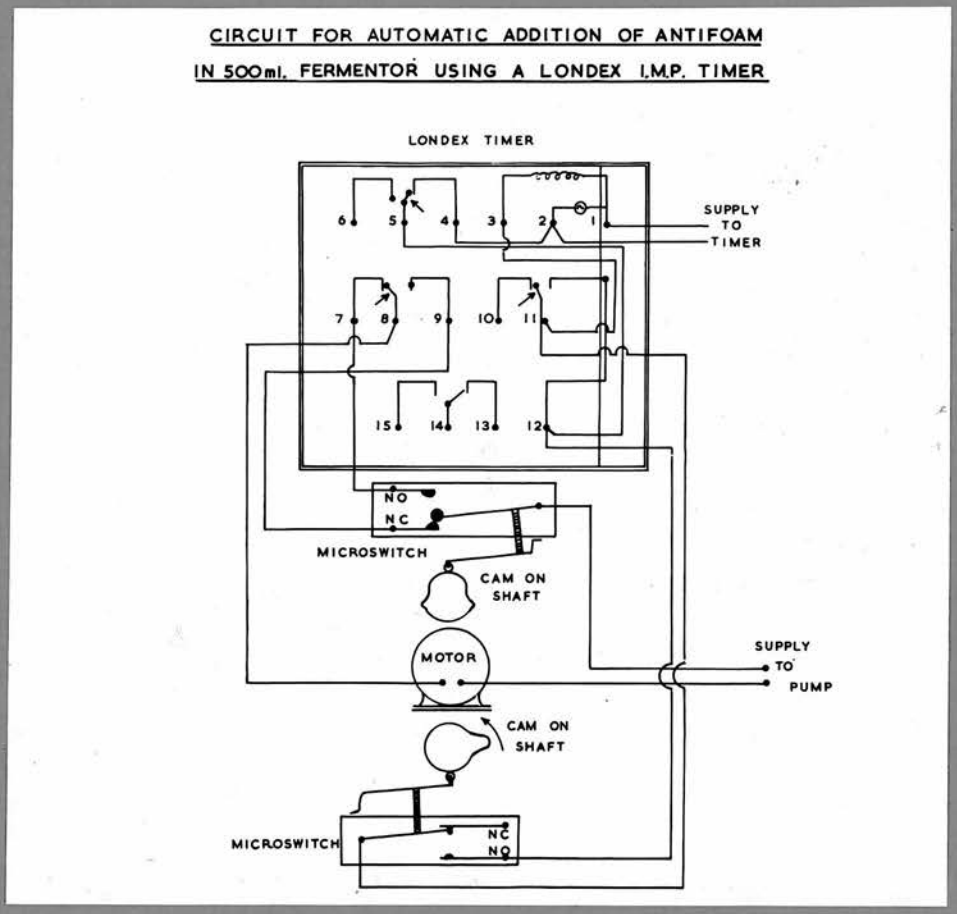

CIRCUIT FOR AUTOMATIC ADDITION OF ANTIFOAM
IN 500ml. FERMENTOR USING A LONDEX I.M.P. TIMER
LONDEX TIMER
6
5
4
3
2
1
SUPPLY TO TIMER
7
8
9
10
11
15
14
13
12
NO
NC
MICROSWITCH
CAM ON SHAFT
MOTOR
SUPPLY TO PUMP
CAM ON SHAFT
MICROSWITCH
NC
NO

Fig. 17.

addition, and the strength of acid or alkali. An accuracy of ± 0.04 pH was standard and often ± 0.02 pH could be achieved by daily supervision. Temperature was not a problem in this system as both electrodes were at 30° and the Dynacap contains a manual temperature setting device. As with temperature, pH recording gives a circular chart if conditions are normal.

Antifoam Addition

The system used is automatic, i.e. antifoam can be added at a given predetermined rate, but it will not detect and control foaming if it occurs. Theorectically, this system 'prevents' foaming, the predetermined rate being in excess and precluding the formation of foam. The metering valve is essentially a very simple peristaltic pump, only three 'fingers' are used, one revolution of the shaft giving one shot of antifoam. The circuit is shown in Fig. 17, being adapted from Elsworth et al. (1956). Instead of a manual switch, a continuously autoresetting timer (Londex Ltd., Type IMP) is used. Thus a shot of antifoam can be added at intervals of from once a minute to once an hour. Silicone MS Antifoam emulsion 'RD' (Hopkinsand Williams) in water (10% w/v) was the only antifoam agent used. Its action is dramatic but brief.

DIAGRAM OF AIR AND MEDIUM SUPPLY LINES

S1, S2.	SAMPLING PORTS	A.F.	ANTIFOAM CONTAINER
F1, F2.	AIR FLOW METERS	A.C.	ACID
P.	PERISTALTIC MEDIUM PUMP	ALK.	ALKALI
M1, M2.	MEDIUM RESERVOIRS	H.	HIGH PRESSURE LINE
B.	BURETTE	G.	GAUGE
F1, F2.	LARGE AIR FILTERS		
F.	SMALL AIR "		
R.	REDUCING VALVE		
N1, N2.	NEEDLE VALVES		
D.	DEVICE TO KEEP MEDIUM INLET FREE OF INFECTION		
SC.	STERILE MEDIUM CONNECTIONS		

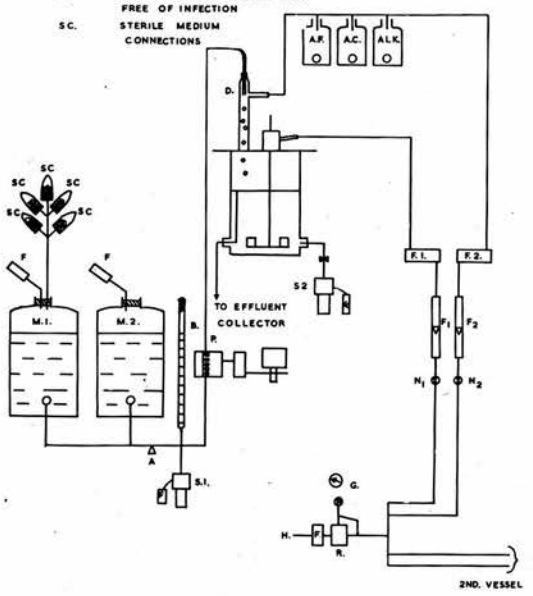


Fig. 18.

Air Supply System

Sterile, metered air flows were supplied to both vessels as shown in Fig. 18. Two air supplies to each vessel are necessary, one to go to the stirrer shaft to aerate the culture, the second ensures that air pressure in the fermentor vessel and acid, alkali, and antifoam bottles is equal, and also prevents back infection of the medium line as shown in the device in Fig. 18. Air is supplied from a high pressure line through a reducing valve (Sunvic Controls Ltd.) giving a steady 3 lbs./sq. ins. This low pressure line is divided into four, each line containing a needle valve and a flow meter (Fischer and Porter Ltd.) calibrated from 50 to 960 ml. of air/minute. Up to this point the air is unsterile, but then each line enters a large tin, 9 ins. in diameter and 3 ins. deep (Crawfords Ltd.), packed with glass wool (Fibreglass Ltd.), the lid being sealed with Picien wax after autoclaving. A standard air flow of 500 ml./min. was adopted for each line.

Medium System

This is also shown schematically in Fig. 18. All line tubing is of silicone rubber tubing (Escorubber Ltd.). The medium is added by a Sigmamotor model T8 peristaltic pump (V.A. Howe Ltd.) driven by a Parvalux 400 r.p.m.

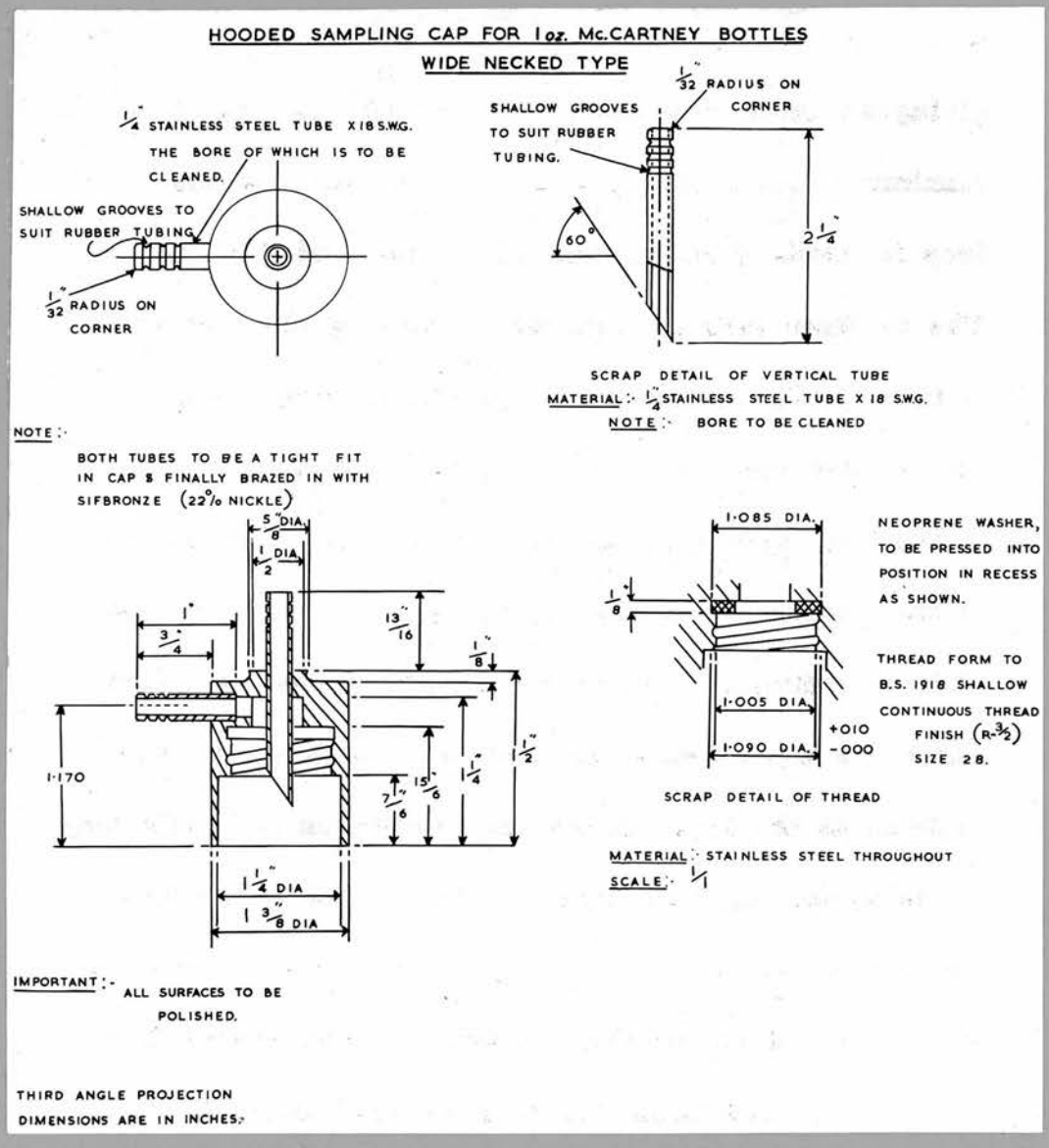

HOODED SAMPLING CAP FOR 1 oz. Mc.CARTNEY BOTTLES
WIDE NECKED TYPE
1/4" STAINLESS STEEL TUBE X 18 S.W.G. THE BORE OF WHICH IS TO BE CLEANED.
SHALLOW GROOVES TO SUIT RUBBER TUBING.
1/32" RADIUS ON CORNER
1/32" RADIUS ON CORNER
SHALLOW GROOVES TO SUIT RUBBER TUBING.
60°
2 1/4"
SCRAP DETAIL OF VERTICAL TUBE
MATERIAL:- 1/4" STAINLESS STEEL TUBE X 18 S.W.G.
NOTE:- BORE TO BE CLEANED
NOTE:-
BOTH TUBES TO BE A TIGHT FIT IN CAP & FINALLY BRAZED IN WITH SIFBRONZE (22% NICKLE)
5/8" DIA.
1/2" DIA.
1"
3/4"
13/16"
1/8"
1·170
15/16"
7/16"
1/4"
1 1/2"
1 1/4" DIA
1 3/8" DIA
1·085 DIA.
1/8"
1·005 DIA.
1·090 DIA.
+·010
-·000
NEOPRENE WASHER, TO BE PRESSED INTO POSITION IN RECESS AS SHOWN.
THREAD FORM TO B.S. 1918 SHALLOW CONTINUOUS THREAD FINISH (R-3/2) SIZE 28.
SCRAP DETAIL OF THREAD
MATERIAL:- STAINLESS STEEL THROUGHOUT
SCALE:- 1/1
IMPORTANT:- ALL SURFACES TO BE POLISHED.
THIRD ANGLE PROJECTION
DIMENSIONS ARE IN INCHES.

Fig. 19.

motor (Parvalux Motors Ltd.) through a Zero-Max gear changer (V.A. Howe Ltd.). The Zero-Max gearbox has a variable output of 0 - 100 r.p.m. Silicone tubing, 2m.m. wall thickness and 6 m.m. bore, is used in the pump giving constant flow rates between 60 ml./hr. up to the maximum tried, 500 ml./hr. Below 60 ml./hr. a second loop is used, 2 m.m. wall thickness and 3 m.m. bore. The medium rate is checked by closing the line at point A in Fig. 18 and timing the medium being pumped out of the burette over a period of 6 min. Each reservoir has a charging device as shown in Fig. 18. The male end of the connection is a glass tube, the female end is of silicone rubber. Before sterilisation, the free ends of the tubes are inserted in test-tubes with cotton wool packing at the top. A sterile connection is easily made by removing the test-tube and fitting the glass tube into the rubber part. After charging, which is done by gravity, the glass rod is carefully drawn out and sealed in a flame. Therefore, each arm can only be used once.

Sampling

A sample is drawn from one of the two ports in the glass culture vessel via the hooded stainless steel sampling port (Fig. 19). This latter port is threaded to fit a

McCartney bottle. A similar sampling point is situated on the medium line.

Effluent

The constant level device is an L-shaped tube $\frac{3}{4}$ ins. above the top of the down-draught tube and coming out of one of the ports in the side of the vessel. Both air and culture depart down this tube. The effluent is collected in a 10 l. aspirator which is kept at approximately 2^{o}. This temperature is achieved by pumping antifreeze solution through 10 ft., of flexible stainless steel tubing (Power Flexible Tubing Co. Ltd.) in the aspirator. A small refrigerator (Townson and Mercer) is used to keep the reservoir of antifreeze at -5^{o}. The air leaves the effluent collector through a cotton wool filter situated on top of the medium pump motor (the heat of the motor prevents condensation in the filter). No attempt is made to empty the effluent collector by aseptic techniques, and there is no reason to suspect that this practice has led to infection of the vessel.

Sterilisation

The apparatus is sterilised by autoclaving. Thus it is divided into three units, the medium reservoirs and medium line, the effluent collector, and the vessel with

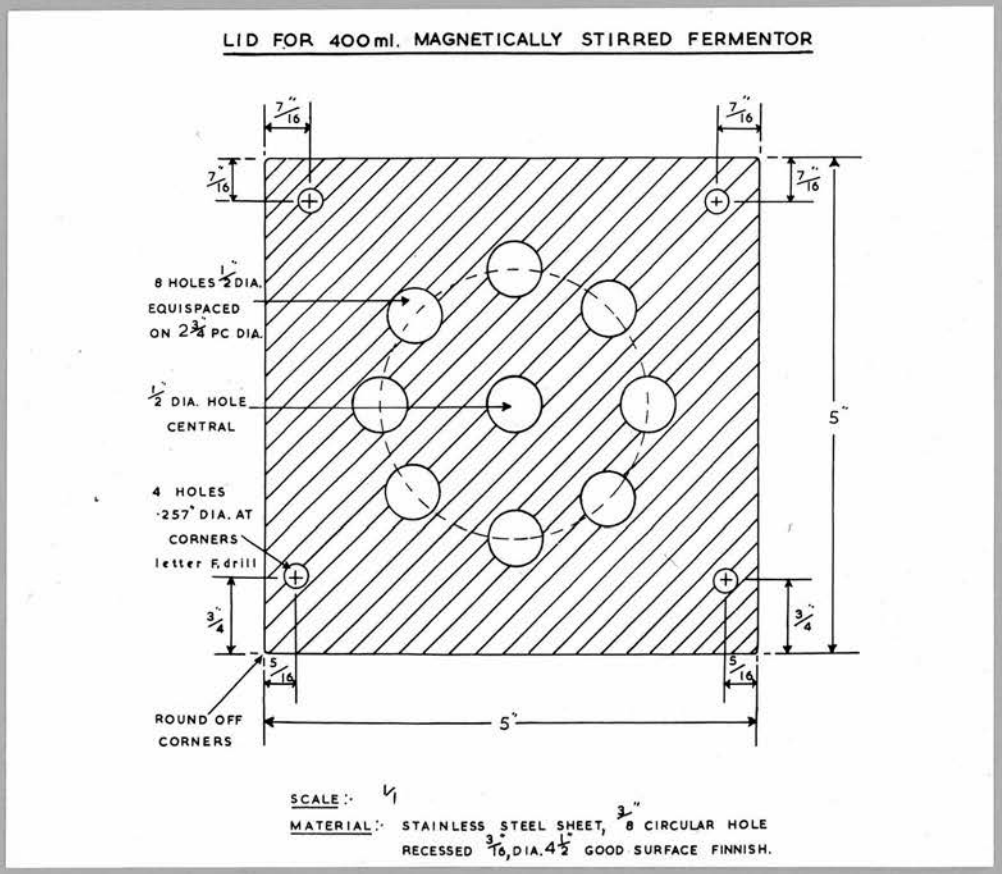

LID FOR 400ml. MAGNETICALLY STIRRED FERMENTOR
7/16"
7/16"
7/16"
7/16"
8 HOLES 1/2" DIA. EQUISPACED ON 2 3/4" PC DIA.
1/2" DIA. HOLE CENTRAL
4 HOLES .257" DIA. AT CORNERS letter F drill
3/4"
3/4"
5/16
5/16
ROUND OFF CORNERS
5"
5"
SCALE :- 1/1
MATERIAL :- STAINLESS STEEL SHEET, 3/8" CIRCULAR HOLE RECESSED 3/16, DIA. 4 1/2" GOOD SURFACE FINNISH.

Fig. 20.

air filters and acid, alkali and antifoam bottles. The acid and alkali bottles are empty. The three sections are joined as described by Elsworth et al. (1956).

Fermenter 2

This chemostat is magnetically stirred and has similar equipment as the other, except that it has no pH control system. They both share the same type of vessel (Fig. 4) and except for minor details, the stainless steel top is also similar (Fig. 20). Instead of a base plate clamping the vessel between it and the top plate, use is made of the rim round the vessel top and a flange (Fig. 21) thus holds down the top, a seal being effected by a neoprene ring. The flange is very necessary in a magnetically stirred vessel where there should be a minimum of distance between the motor driven magnet outside the vessel and the following magnet in the vessel. As before there is a hollow shaft, in the centre port (Fig. 22) of the top plate, down which air passes to the impeller. The shaft also functions to locate the magnetic impeller in the centre of the vessel. The impeller (Fig. 23) is similar to the previous one except that the blades are smaller and underneath is fixed a U-type

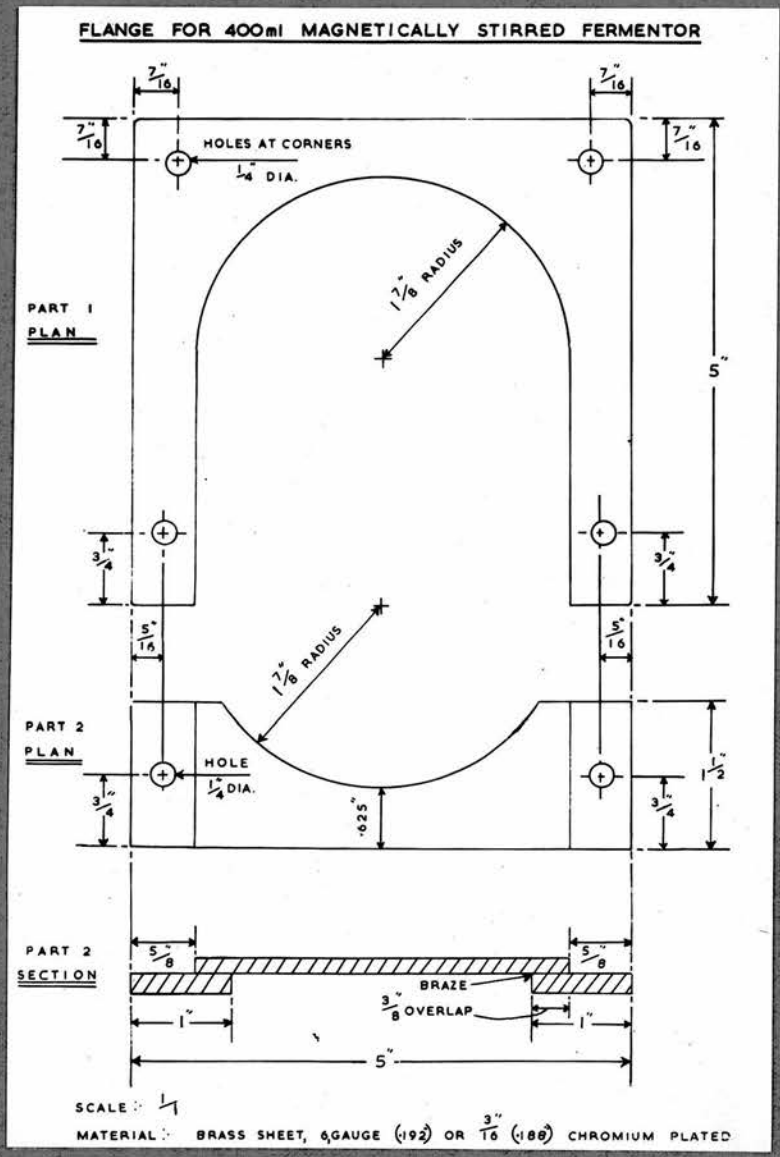

FLANGE FOR 400ml MAGNETICALLY STIRRED FERMENTOR
7/16"
HOLES AT CORNERS
1/4" DIA.
1 7/8" RADIUS
5"
PART 1
PLAN
3/4"
5/16"
PART 2
PLAN
HOLE
1/4" DIA.
.625"
1 1/2"
PART 2
SECTION
5/8"
BRAZE
3/8" OVERLAP
1"
5"
SCALE : 1/1
MATERIAL : BRASS SHEET, 6 GAUGE (.192") OR 3/16" (.188") CHROMIUM PLATED

Fig. 21.

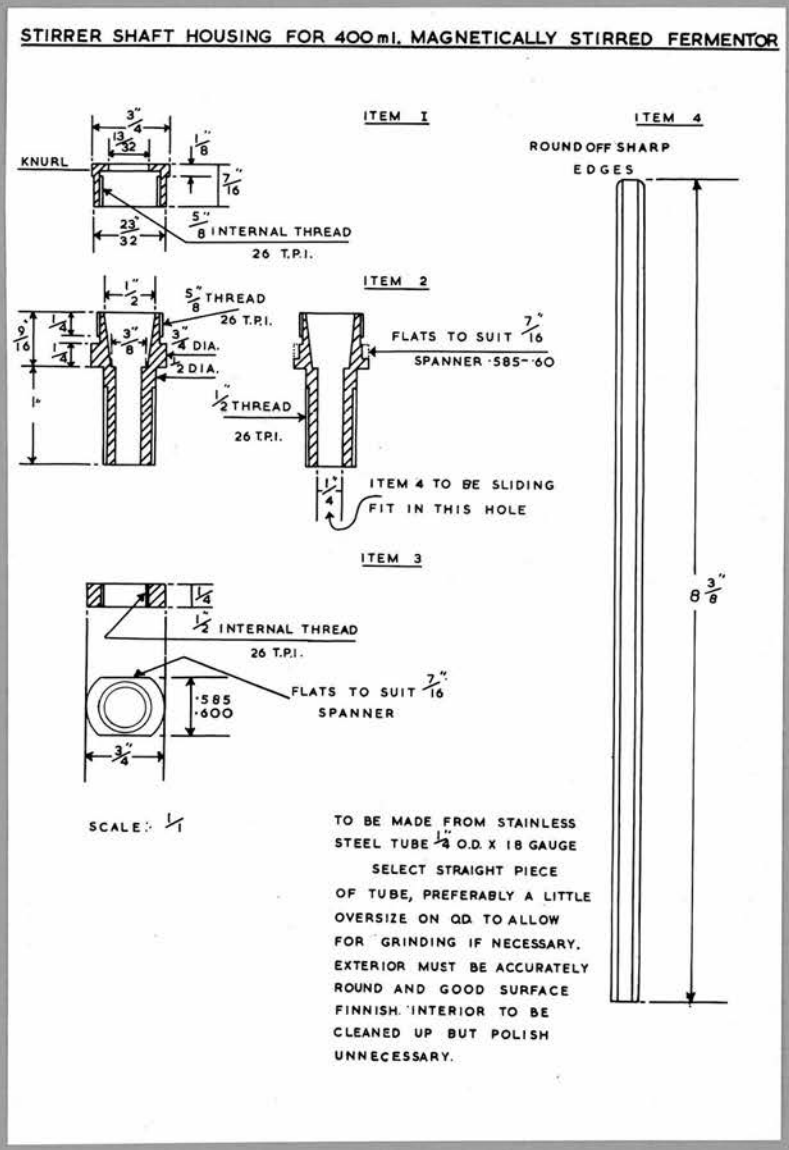

STIRRER SHAFT HOUSING FOR 400 ml. MAGNETICALLY STIRRED FERMENTOR
ITEM 1
KNURL
5/8 INTERNAL THREAD
26 T.P.I.
ITEM 2
5/8 THREAD
26 T.P.I.
3/4 DIA.
1/2 DIA.
1/2 THREAD
26 T.P.I.
FLATS TO SUIT 7/16
SPANNER ·585-·60
ITEM 4 TO BE SLIDING
FIT IN THIS HOLE
ITEM 3
1/2 INTERNAL THREAD
26 T.P.I.
FLATS TO SUIT 7/16
SPANNER
·585
·600
ITEM 4
ROUND OFF SHARP
EDGES
8 3/8
SCALE: 1/1
TO BE MADE FROM STAINLESS
STEEL TUBE 1/4 O.D. X 18 GAUGE
SELECT STRAIGHT PIECE
OF TUBE, PREFERABLY A LITTLE
OVERSIZE ON O.D. TO ALLOW
FOR GRINDING IF NECESSARY.
EXTERIOR MUST BE ACCURATELY
ROUND AND GOOD SURFACE
FINNISH. INTERIOR TO BE
CLEANED UP BUT POLISH
UNNECESSARY.

Fig. 22.

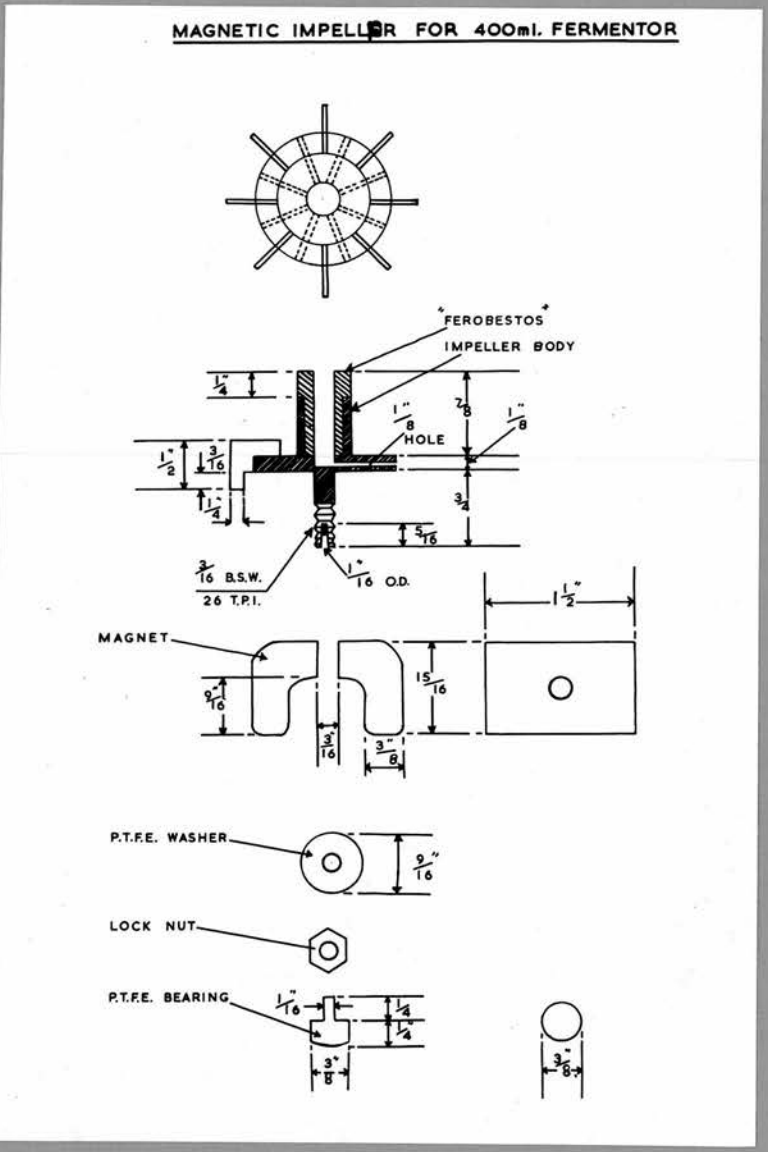


Fig. 23: Dimensions of the impeller are similar to those shown in Fig. 8a unless otherwise indicated.

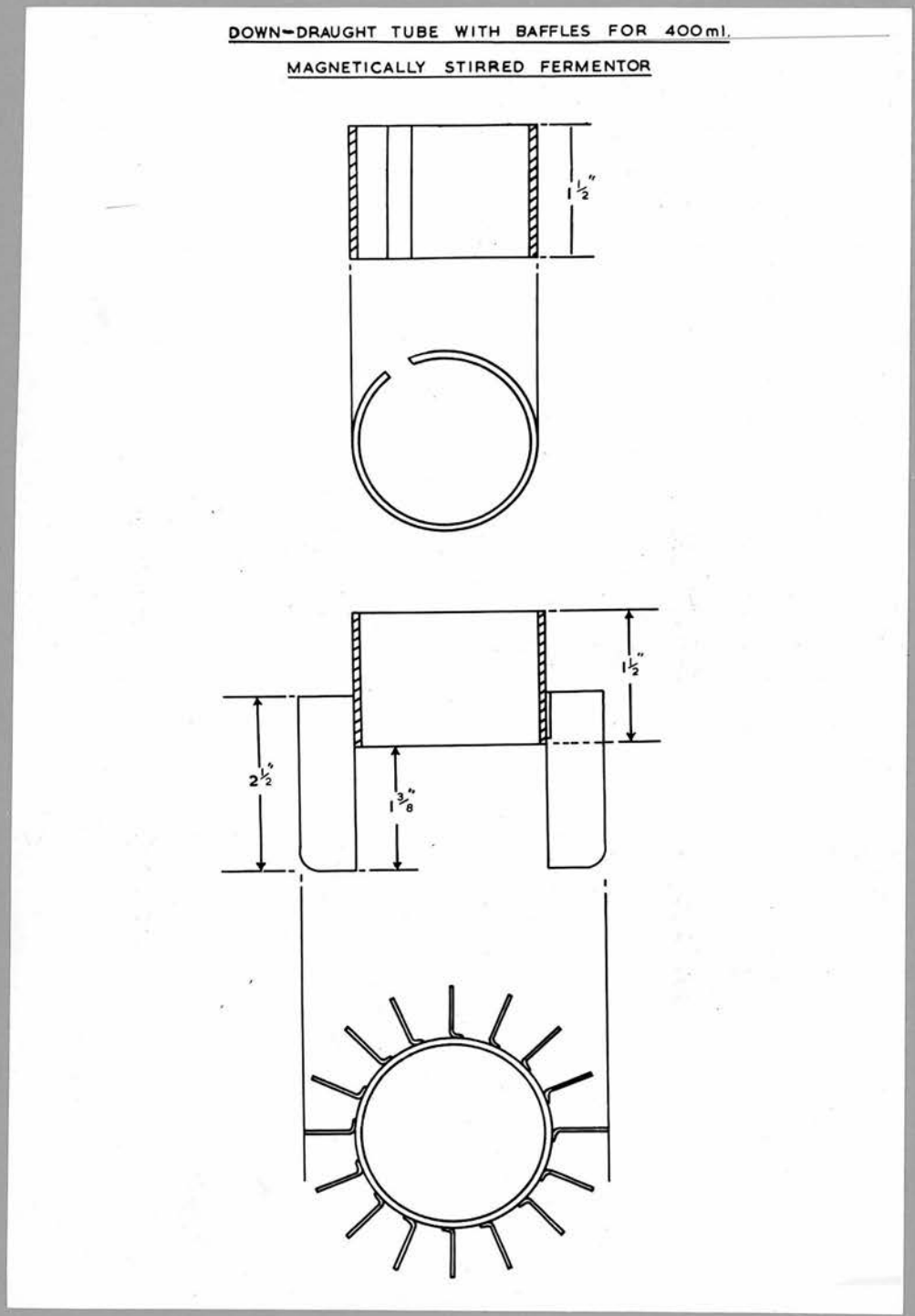


Fig. 24: Dimensions of the down-draught tube are similar to those shown in Fig. 10 unless otherwise indicated.

magnet. The whole impeller assembly spins on a Fluon (James Walker & Co. Ltd.) bearing at the bottom, and revolves round a Ferrobestos bearing at the top. To prevent corrosion, the magnet is coated with an epoxy resin which will withstand autoclaving and wear (Gittings & Hills Ltd.).

The baffling system (Fig. 24) is modified to allow for the difference in impeller size. The operating volume is 400 ml. or less, any more leads to a break in the magnetic coupling. Similarly, a 1000 r.p.m. is the maximum safe speed for the coupling at this volume. A stirrer speed of 920 r.p.m. and an air rate of 400 ml/min. are routinely used. The magnets are standard patterns (James Neill & Co. Ltd., Eclipse 812 B, C).

Temperature Control

The system used in this chemostat is much simpler and cheaper. The electrical circuit is shown in Fig. 25. A contact thermometer operating through an electromagnetic relay (G.H. Zeal Ltd.) supplies power to a heating element. When the mercury in the thermometer reaches the pre-determined setting it breaks the supply to the heater, and when the mercury drops below the setting it again makes

the heater circuit. The heater operates against a cold finger in which a very slow flow of cold water circulates all the time. This heater consists of the resistance wire from a 25 watt soldering iron element. Wet asbestos paper is wound round the bottom 4 ins. of a $^{3}/_{16}$ ins. o.d. hollow silica glass tube 9 ins. long. The resistance wire is wound round the wet paper keeping each turn apart, one lead going up the inside of the tube, the other on the outside. This heating probe fits inside a jacket $^{5}/_{16}$ ins. i.d. of silica glass tubing. During autoclaving the heater probe is removed. The contact thermometer, supplied by the makers of the relay, has four predetermined temperature controlling points, 25°, 30°, 35°, 37°, also fits inside a jacket in one of the ports and consequently does not need autoclaving either. The degree of control with this system is at least ± 0.2°.

Temperature Recording

As described in the other fermentor.

pH Control

The buffering capacity of the medium is used to control pH.

Antifoam Addition

The antifoam addition system is similar to that

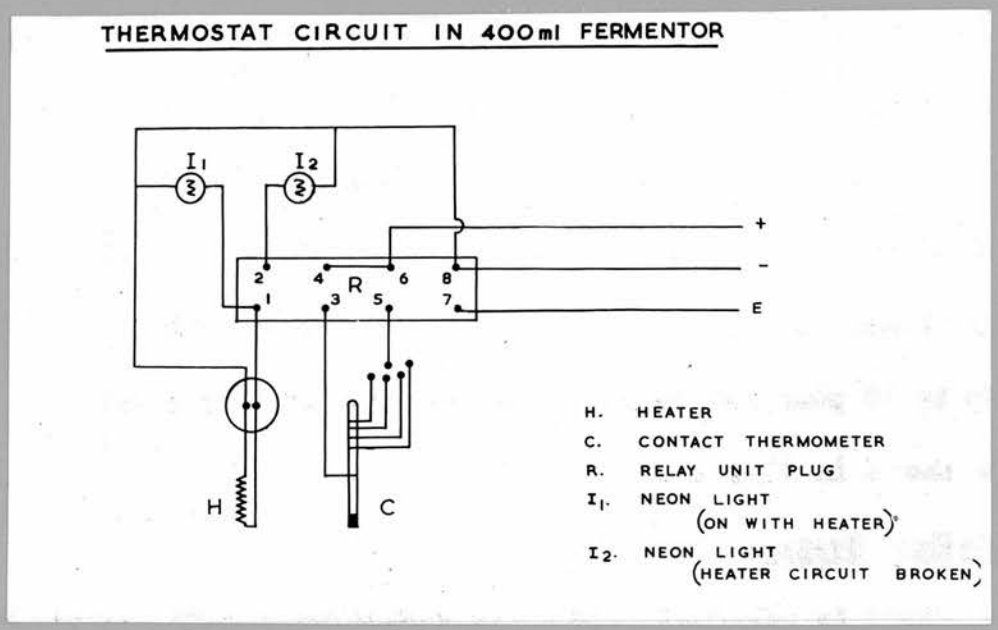

THERMOSTAT CIRCUIT IN 400ml FERMENTOR
I1
I2
2
4
R
6
8
1
3
5
7
+
-
E
H
C
H. HEATER
C. CONTACT THERMOMETER
R. RELAY UNIT PLUG
I1. NEON LIGHT (ON WITH HEATER)
I2. NEON LIGHT (HEATER CIRCUIT BROKEN)

Fig. 25.

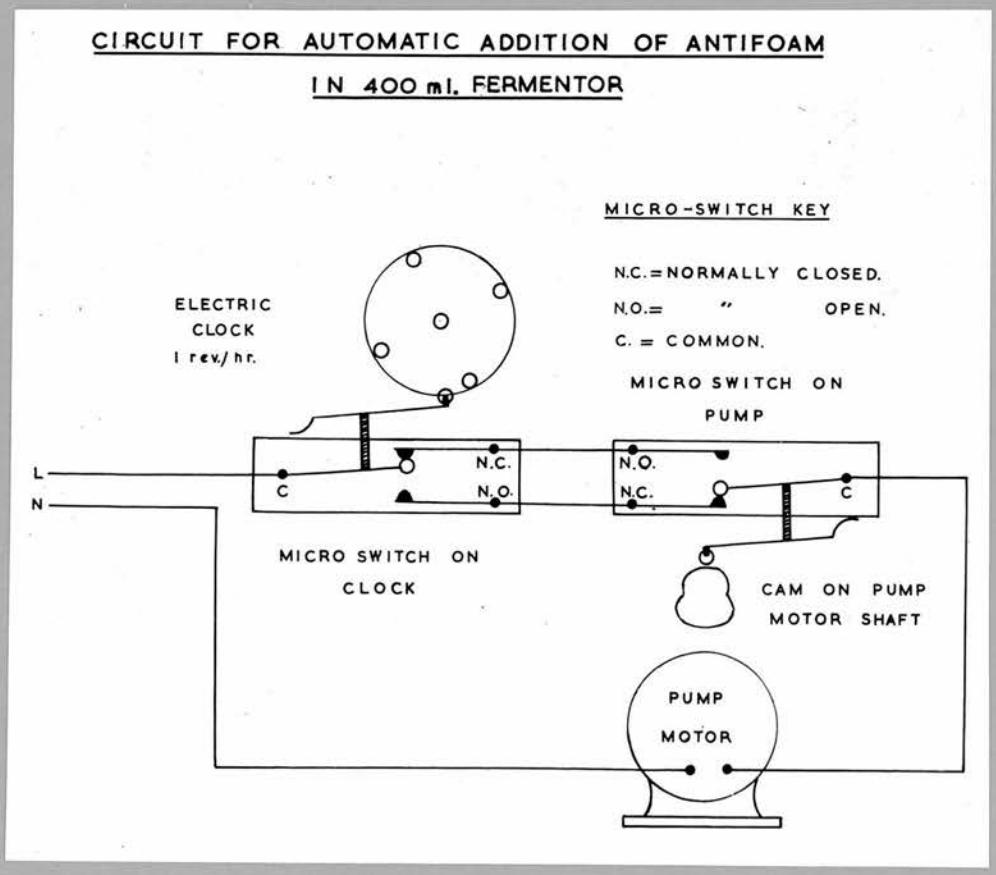

CIRCUIT FOR AUTOMATIC ADDITION OF ANTIFOAM
IN 400 ml. FERMENTOR
MICRO-SWITCH KEY
N.C.=NORMALLY CLOSED.
N.O.= " OPEN.
C. = COMMON.
ELECTRIC
CLOCK
1 rev./hr.
MICRO SWITCH ON
PUMP
L
N
C
N.C.
N.O.
N.O.
N.C.
C
MICRO SWITCH ON
CLOCK
CAM ON PUMP
MOTOR SHAFT
PUMP
MOTOR

Fig. 26.

already described. However, instead of the Londex timer, a synchronous motor giving a speed of 1 rev. per hr. to a shaft bearing a tufnol wheel is used. A pin on the tufnol wheel strikes a microswitch once an hour giving one revolution of the pump shaft and one shot of antifoam. Up to 10 pins can be added to the wheel. The circuit is shown in Fig. 26.

Medium System

This is identical to the one described for Fermentor 1.

Assembly

A photograph of Fermentor 2 is shown in Fig. 27.

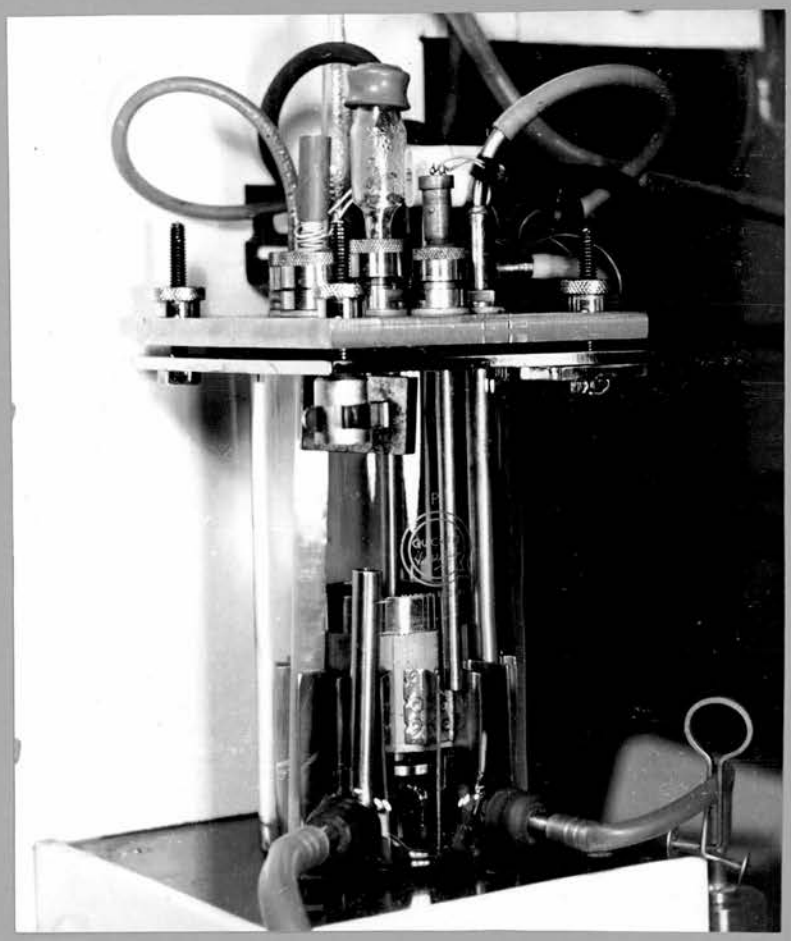


Fig. 27. Fermentor 2.

V

METHODS

METHODS

Bacillus megaterium Strain KM, kindly supplied by Dr. K. McQuillen, had been trained to grow on a synthetic medium containing a basal salts solution (McQuillen, 1955). It is asporogenous and also lysozyme sensitive, thus facilitating the preparation of cell free extracts for enzyme studies. The organism was routinely kept as a freeze-dried culture, the parent strain always being used for the start of each run with a different limiting nutrient.

Temperature

All growth, washed-cell suspension and cell-free extract experiments were carried out at 30°.

Media

The constituents present in a fully nutrient medium are shown in Table 2.

Table 2

Details of Media used:

Constituent	Medium for Fermentor I gm/l.	Medium for Fermentor II gm/l.
Na_2HPO_4	0.8	9.0
$NaHPO_4$	0.1	0.25
NaCl	2.0	2.0
KCl	1.0	1.0
Na_2SO_4	0.5	0.5
$MgCl_2\ 6H_2O$	0.1	0.1
$MnCl_2\ 4H_2O$	0.01	0.01
NH_4Cl	0.5	1.5
Glucose	10.0	10.0
Citric acid	0	0.2

The organism has been cultured continuously to date under limiting glucose, nitrogen, sulphate or potassium. Carbon limitation with compounds other than glucose was also carried out using glycerol, lactate, or succinate. It has also been cultured under limiting glucose plus 0.1 M. acetate and limiting nitrogen plus 0.1 M. acetate. The concentrations of these substances or ions used for limiting purposes are shown in Table 3.

Table 3

Concentration of the limiting nutrients

Nutrient	Limiting Concentration		
	gm/l.	ug/ml.	Molarity in m moles.
Glucose	2.0	2000	11.1
Glycerol	2.04	2040	22.2
Sodium Lactate (70% W/W)	3.56	2000 (Lactic acid)	22.2
Sodium Succinate $6H_2O$	4.5	1970 (Succinic acid)	16.7
NH_4CL	0.325	85(N)	6.06
Na_2SO_4	0.015	3.38(S)	1.06
KCl.	.00625-.075	-	-

All carbon limitations contain the same number of carbon atoms / l. of culture. The potassium deficiency required a different concentration at each dilution rate.

The pH of Fermentor I was maintained at 7.3 and the pH of the medium flowing in was 7.3. To keep the culture at pH 7.3 in Fermentor 2 the ingoing medium was buffered to pH 7.7 - 7.9, depending on the dilution rate. The medium was sterilised in 20 l. batches, being divided into three parts, a 20 l. aspirator and two 2 l.

aspirators joined together by silicone rubber tubing and T-pieces. The 20 l. aspirator contained 17.5 l. of distilled water plus the carbon source and citric acid, and one of the 2 l. aspirators the phosphates in 1600 ml. of water, and the other, the salts in 1600 ml. of water, the 700 ml. excess water being lost during autoclaving. Autoclaving was carried out for 90 mins. at 13 lbs/sq.ins. The citric acid has a dual role in the medium for Fermentor 2, one to reduce the pH of the glucose solution to prevent caramelisation during autoclaving, and secondly to complex the magnesium and manganese ions which would otherwise precipitate as phosphates during the quite often long periods of standing at pH 7.9 before use. Above this pH the citrate lost its sequestering action. In the medium for Fermentor 1, one drop of concentrated phosphoric acid was added to reduce the pH during autoclaving.

Inoculation of Vessel

This was achieved by 2 mls. of culture, from an overnight shake flask of glucose broth through the Suba-seal bung in the inoculation port. Inoculation from shake flasks of synthetic medium was nearly always unsuccessful.

Turbidimetric and Spectrophotometric Measurements

In procedures requiring either type of measurement a Unicam SP 500 or 600 spectrophotometer was employed with cuvettes of 1 cm. light path and 4 ml. capacity.

Criteria of Steady State Growth

The simplest routine method of checking constant growth conditions in the vessel was to determine the turbidity of the culture. Thus, after a small sample (5 - 10 ml.) was withdrawn, 1 ml. was diluted with sufficient 1% (W/V) NaCl so that the turbidity, which was measured at 530 mu, fell on the linear portion of the optical density curve. The whole operation was carried out quickly as it was found that leaving the sample, whether diluted or not, for any length of time, led to an increase in turbidity. Steady-state conditions were judged to be reached when the turbidity values were constant for more than 48 hrs., or for at least 10 generations, whichever was longest. Providing large samples were not taken during the day, turbidity checks were carried out twice daily, morning and evening. When it was judged the steady state had prevailed for long enough, one large sample was drawn and split up immediately according to the analysis

to be performed. A resumé of the treatment of a typical sample is given in Table 3a.

Table 3a.
Details of how a large sample was treated on removal from the fermentor

Sample for	Volume (ml.)	Procedure
Washed cells for dry weight polysaccharide, glycogen, nitrogen, phosphorus analyses.	150	Washed 3 times in 1% (W/V) NaCl. Culture supernate kept and stored at -20°. Washed cells made up to a known volume, the dry weight determination carried out and the rest stored at -20°.
PHB analyses	20	4x5 ml. samples, centrifuged and the cell material suspended in alkaline hypochlorite.
Nucleic acid analyses	20	4x5 ml. samples, centrifuged in cold room, cells stored at -20°.
Total cell count	5-10	Formalised and stored at 2°.
Degradation of PHB and polyglucose in intact washed cells	150	See text of results.
Heat fixed smears for staining.		

In all cases where cells are referred to as being centrifuged, a Servall SS-1 Superspeed Angle Centrifuge was used giving a Relative Centrifugal Force of 1,230.

Determination of Dry Weight

Bacteria (20 - 25 mg. dryweight) were centrifuged from a measured volume of bacterial suspension from the culture vessel, washed three times with 1% (W/V) NaCl, the first wash containing 0.5% (W/V) formaldehyde. The washed cells were dried at 105° for 16 - 20 hr. in previously dried and weighed Petri dishes. Similar Petri dishes containing an equal volume of 1% (W/V) NaCl were also dried and weighed. Each determination was carried out in triplicate, the results averaged, and the dry weight of bacteria obtained by subtracting the weight of NaCl from the weight of bacteria plus NaCl. Individual weighings did not vary more than 0.75% of the mean.

Determination of PHB

PHB in growing cultures was measured by the method of Law and Slepecky (1961). Briefly, quadruplicate 5 ml. samples of culture were centrifuged and the cells resuspended for 6 hr. in the alkaline hypochlorite reagent of Williamson and Wilkinson (1958). The suspension of lipid granules which remained was centrifuged and washed sequentially with water, acetone and alcohol. The dry residue was extracted with chloroform, filtered, and the filtrate then evaporated to obtain the purified polymer.

This was subsequently heated with 10 ml. of concentrated sulphuric acid in a boiling water bath for 10 min. and then quickly cooled to room temperature. Crotonic acid, which is produced by this treatment, shows an absorbtion peak at 235 mμ; the principle of this method (Slepecky and Law, (1960) is that the UV-absorbtion maximum of α,β -unsaturated acids undergoes a strong bathochromic shift when concentrated sulphuric acid is employed as a solvent. The solution of crotonic acid was further diluted with concentrated sulphuric acid until it contained approximately 2 - 3 ug. per ml. The absorbtion values of the quadruplicate samples did not vary by more than 4% of the mean value. This means that a sample containing a mean value of 5% of the dry weight as PHB might vary by $\pm$ 0.2% whereas one containing a mean value of 15% of the dry weight as PHB might vary by $\pm$ 0.6%.

Experiments involving the determination of the amount of self-degradation of the polymer by washed cells were always measured by the turbidimetric method of Williamson and Wilkinson (1958). This method involved measuring the turbidity at 530 mμ of an alkaline hypochlorite suspension of lipid granules. The cells containing the lipid granules had been allowed to degrade their polymer

reserves after being suspended for various times in medium without a carbon source in a vigorously shaken flask which allowed moderate oxygen transfer rates. For example, 100 ml. of culture was centrifuged, washed once at 30°, and suspended in medium minus a carbon and nitrogen source at 30°. Samples (5 ml. in duplicate) were taken every 20 min. for 80 min., the cells centrifuged and suspended in alkaline hypochlorite reagent (5 ml.) for 12 - 16 hr.

Determination of Total Nitrogen

This was determined by the micro-Kjeldhal procedure. Samples, which normally contained from 200 - 400 ugN, were digested with a mixture of K_2SO_4, $CuSO_4$, and Se catalyst on an electrically heated rack. Steam distillation was carried out in a modified form of the apparatus described by Scandrett (1953) and latterly in a Markham still. The ammonia was absorbed in boric acid and backtitrated against 0.01 N sulphuric acid using a mixed indicator of methylene blue and methyl red. All samples were assayed in duplicate.

Determination of Polysaccharide

Total polysaccharide was determined in duplicate by the anthrone method of Dreywood (1946) as modified by

Fairbairn (1953) using 0.1% (W/V) anthrone in 72% (W/V) sulphuric acid. The suspension of organisms (2 ml.), contained in a 150 m.m. x 25 m.m. boiling tube, was cooled in ice-water while anthrone reagent was slowly added. The tube was then heated at 100° for 8 min. and cooled in water. The colour was measured at 625 mμ. Blanks and glucose standards (containing 100 μg) were included in each set of determinations. Duplicate determinations did not vary by more than 3% of the mean.

Detection and Determination of Polyglucose

Alkali soluble, alcohol insoluble polysaccharide was extracted by the method of Good, Kramer and Somogyi (1933) as developed by Palmstierna (1956). This method gave recoveries of 90 - 95% using a commercial preparation of glycogen (British Drug Houses Ltd.). The isolated, washed polysaccharide was hydrolysed for 3 hr. with 0.6 N HCl in a stoppered tube at 100°. The resulting glucose in the hydrolysate was determined by glucose oxidase (Hugget and Nixon, 1956) using the blood sugar method and enzyme preparation of C.F. Boehringer and Soehne, GmbH. The products of hydrolysis were also subjected to single dimensional descending paper chromatography using Whatman No. 1 paper with two solvent systems:-

a. Ethyl acetate : pyridine : water, 2: 1 : 2, (Jermyn and Isherwood, 1949).

b. Ethyl acetate : acetic acid : water, 3 : 1 : 3, (Jermyn and Isherwood, 1949).

All solvents not of analytical reagent quality were redistilled prior to use. Chromatograms were run for 24 hr. at room temperature in both solvent systems. The spots were applied using micro-pipettes prepared from capillary tubing. A glucose standard was included in all chromatograms. Solvents were allowed to evaporate from the papers at room temperature in a fume cupboard. Reducing sugar spots were detected by spraying with p-anisidine hydrochloride (Hough, Jones and Wadman, 1950).

Glucose Yield Constant and Glucose Oxidation Rate

To calculate the glucose yield constant (GYC) and the glucose oxidate rate (GOR) it was necessary to determine the glucose remaining in the spent culture. The glucose determination was initially carried out by the anthrone method but later the glucose oxidase method of Hugget and Nixon (1956) was used as it was easier to perform and less liable to variation.

Extraction of Nucleic Acids

Three extractions with 0.5 N perchloric acid at 70° for 15 min. were found necessary to remove all the DNA and RNA, the supernatants being combined and made up to a suitable volume for colourimetric sugar tests. Extractions were always carried out on quadruplicate samples.

Determination of Deoxyribose

Deoxyribose was determined by the diphenylamine reaction of Dische (1930) as modified by Burton (1956). The assay was performed using 2 ml. of extract and 4 ml. of reagent, all blanks and standards were made up in 0.5 N perchloric acid. DNA from herring sperm (British Drug Houses Ltd.) was routinely used as standard (46.5 µg/ml.). The intense blue colour, developed in 16 - 20 hr. at 30°, was read at 600 mµ. The absorbtion values seldom varied by more than 2.3% of the mean value.

Determination of Ribose

Ribose was determined by the Bial reaction, with orcinol and ferric chloride in HCl, using the method described by Kabat and Meyer (1961). The reagent reacts with all pentoses and will almost certainly give a higher answer than the true value. The extract (1 ml.), mixed with 5 ml. of reagent and 0.4 ml. of 10% (W/V) orcinol solution in ethanol, was heated for 6 min. at 80°. Standards containing 24 µg. ribose were also run. The reaction mixture was chilled in ice-water and kept at 0° until the absorbtion was measured at 670 mµ. As orcinol reacts only with the purine ribose, a factor of 4.91 (Schaechter et al., 1958) was used to convert ribose to RNA. Absorbtion readings regularly varied by as much as 4.1% of the mean value from four similar extracts.

Volatile Acids

Total volatile acids were steam distilled in a Markham still (Friedmann, 1939). The distillate was collected and titrated immediately against CO_2 free 0.01N NaOH using phenyl red as indicator.

The titrations obtained by this method were unaltered by refluxing the sample in presence of mercuric

salts and then steam distilling, demonstrating that formic acid was absent. Acetic acid was identified by the lanthanum nitrate spot test. In view of the fact that no other volatile acid was identified in the distillate it is calculated and described as acetic acid in the results section.

Keto Acids

The keto acids occuring in the culture supernate were identified, after conversion to their 2, 4 -dinitro - phenylhydrazones, by paper chromatography (Cavallini and Frontali, 1954). Only two were found, always in admixture, namely pyruvate and 2-oxoglutarate. They were determined by the method of Koepsell and Sharp (1952).

Determination of Phosphorus

Inorganic and total phosphorus were determined by Allen's method (1940) using 2% $FeSO_4$ (W/V) as reducing agent.

Fractionation of Phosphorus Compounds

Initially, fractionation was carried out according to the method of Juni et al. (1958) which is essentially an adaptation of the Schmidt and Thaunhauser technique. Later, the method of Harold (1960) was adopted because it appeared to be more specific for polyphosphate and was less tedious and simpler to perform. Essentially, it is a variation of

the Schneider (1945) perchloric acid fractionation technique.

Briefly this was carried out as follows, 40 ml. of culture was washed once in 1% (W/V) NaCl and then once in acetone. The cell material was extracted with cold 0.5 N perchloric acid, twice for 15 min. at 0°, then with ethanol alone, boiling ethanol ether (1 : 2) and finally with 0.5 N perchloric acid at 70°. The following determinations were carried out on the combined cold perchloric acid extract; orthophosphate (Pi), total phosphate ($P_T$ot), and the acid labile phosphate (Polyphosphate) remaining after the nucleotides had been removed on activated charcoal which had been washed with perchloric acid to remove impurities of phosphate. The organic (nucleotide) phosphorus fraction (Po) defined as $P_T$ot - (Pi + soluble polyphosphate) was determined by difference. The hot perchloric acid extracts contained the insoluble polyphosphate together with the nucleic acids. The latter were removed by adsorbtion on activated charcoal and determined by difference.

Determination of ATPase Activity

The ATPase activity was measured by the method of Slade, Doughty and Slamp (1954). Cell free extracts of B. megaterium were prepared by concentrating 40 ml. of culture

to 3 ml. in a test-tube, 50 m.m. x 10 m.m. The suspension, partially immersed in a beaker of ice-water was treated for 20 min. with an MSE-Mullard ultrasonic disintegrator using a $\frac{1}{4}$in. diameter probe. The crude extracts were dialysed against 0.1 M KCl plus 0.1% (W/V) $MgSO_4$ for 24 hr.

The method involved measuring the inorganic phosphate liberated by this extract in presence of ATP or ADP. The extract (0.2 ml.) was incubated for 20 min. at 30° with 2 μ mole of ATP or ADP (CF. Boehringer & Sohne, GmbH), 2 μ mole of $MgSO_4$ and 20 μ mole of Tris-HCl buffer pH7, the total volume of the reaction mixture being made up to 1 ml. wtth distilled water. Zero time controls and controls on reagents and the enzyme preparation were included, otherwise the experiment was similar to that described by Slade et al. (1954).

Measurement of Sulphite Oxidation Rates

Sulphite oxidation rates were carried out according to the method of Cooper et al. (1944) as described by Elsworth et al. (1957).

Measurement of Doubling Time in Batch Culture

This measurement involved determining the maximum growth rate of a sample of cells transferred from a steady state in the culture vessel to a complete medium where all

nutrients were in excess. The cell material from the continuous culture vessel was immediately transferred into 100 ml. of complete medium at 30° in a 250 ml. conical flask. The amount of cell material added was sufficient to give a turbidity reading of 0.03 - 0.06 turbidity units when added to the contents of the flask. The flask was shaken vigorously on a shaker with a 'push-pull' action. No air was bubbled through the contents of the flask. Oxygen transfer rates were not measured in this system but it is unlikely they are anywhere near as high as in the Fermentor vessels. After 1 - 2 hr. the turbidity was measured at known intervals of approximately 10 - 30 min. depending on the growth rate of the cells. The turbidity readings (5 - 7) were plotted on semi-log graph paper against time in min. The plots obtained were straight lines, the time required for the turbidity to double was taken as the mean doubling time of the culture and the specific growth rate calculated from:-

$$\text{Doubling time } (t_d) = \frac{\log 2}{\text{Specific Growth Rate}}$$

$$t_d = \frac{0.693}{D}$$

Total Cell Count

Counts were made in a 'Thoma' counting chamber using the 'high dry' magnification of a phase contrast microscope.

Staining Methods:-

Lipid Granules The method of Burdon (1946) was used, but basic fuchsin replaced safranin as the counter stain. The inclusions nearly always appeared black, sometimes bluish black, while the cytoplasm stained red. The best results were obtained by leaving the Sudan Black B on the slide for as long as possible, without, however, letting the Sudan Black dry out.

Volutin Inclusions These were stained by Leybourn's (1924) modification of Albert's method. The inclusions appear black, and the cytoplasm green.

Gram Staining Technique was carried out as described in the 'Handbook of Bacteriology' (Mackay and McCartney).

VI

RESULTS

RESULTS

Behaviour and Performance - Fermentor 1

During the first run with this vessel, a series-wound electrical motor was used for stirring and the speed was controlled by a rheostat. When carrying out sulphite oxidation studies, it was not apparent that the speed of the motor would vary considerably according to the volume stirred. This was a self-aggravating system leaving little but foam in the vessel at low dilution rates. The series-wound motor was, therefore, replaced as soon as a suitable constant speed induction motor of 1400 r.p.m. could be obtained.

Antifoam was another incalculable factor in this system and its use was abandoned in the second run as soon as it was realised that dry weights of 1.5 mg. per ml. and less were sufficient for most needs. This was fortunate since the antifoam contained unknown quantities of mineral ions such as K^+ and SO_4^{2-} which would seriously have handicapped later runs where these ions were the limiting substrates. The reduction to 1.5 mg. per ml. and less was unfortunate because a vessel with such high oxygen transfer rates was capable of supporting ten times as many cells.

It was soon discovered that an important factor controlling the operating volume was the setting of the height of the downdraught tube and the height of the overflow tube above it. When the difference in height between the two was correct (approximately $\frac{3}{4}$ins.) foam breaking and aeration was maximal. However, using water to judge these settings was not equivalent in its physical properties to a glucose solution containing Bacillus megaterium. As a result it was difficult to reproduce precisely identical settings in each run, and the volume varied between 400 - 500 ml. or 500 - 600 ml. Of course, it was quite constant at any dilution rate, ($\pm$ 5 ml.) and the outside of the vessel was calibrated to give the volume of the culture when the stirrer was stopped. Every time the rate of medium flow was checked the volume in the vessel was recorded.

All the other systems, including temperature and pH control, worked remarkably well for long periods with relatively little attention. Air-locks sometimes blocked the acid and alkali lines because the gravity feed was insufficient if the reservoirs were low and had no head of liquid. With experience, however, the difficulties were

usually diagnosed before a disaster occurred. The rate of flow of cooling water had occasionally to be checked to prevent the infra-red lamp from being on for excessive periods.

At the end of 7 - 9 weeks it was usually found that the stirrer gland started to leak, indicating that sterility was broken. This was probably due to two causes, either the shaft was not perfectly round or was not perfectly straight. The latter fault created a slight whipping of the impeller causing, first, wear on the lower 'Ferrobestos' bearing in the gland and finally, wear on the oil seal. It was only after such a leak that the vessel contents became contaminated. However, the worst source of infection was always through the presence of moulds in the medium containers. We are inclined to attribute this rather to the fact that there were no flanges to hold the rubber bungs tightly in position in the necks of the two aspirators, than to faulty autoclaving. The small, cotton wool packed, air filters on the medium containers may also have been at fault because this type of infection never occurred during the first few weeks of a run.

Some care had to be taken to ensure that the bath

of antifreeze did not get too cold; if this happened, the culture in the effluent collector solidified, blocking the outlet point, and it sometimes took as long as 24 hrs. to thaw out.

Behaviour and Performance - Fermentor 2

Much the same applied to this vessel in regard to the down-draught tube. The difference in height between the overflow tube and the top of the down-draught tube was also critical in achieving foam-breaking conditions and circulation of the culture in the vertical as well as in the horizontal plane. In the two runs described in this thesis, the operating volume lay between 320 and 400 ml.

Infection did not occur directly in the vessel indicating that the magnetically stirred system (when a strong enough magnetic coupling can be incorporated to give good stirring) is as free of infection risks in practice as theory predicts it should be. The equipment in this chemostat was much easier to set in operation and ran without maintenance for indefinitely long periods, mainly because it had no pH control system. The lack of such a system was also the main drawback to this fermentor, because growing an acid-producing bacterium in excess glucose under mineral limitation and attempting to maintain

the pH at 7.3, has shown that some conditions are virtually impossible to meet without resorting to drastically heavy buffering or, reducing the yield of organisms below a point where there is insufficient material to harvest.

Choice of Buffering Agents

In Fermentor 1 it was necessary to have some buffering capacity to absorb the addition of acid or alkali. Ideally, such a buffer should have no direct physiological action on the cells. An initial investigation using Tris (hydroxymethylaminomethane) under nitrogen limitation showed a nitrogen content of the cells, 12% higher than was added as NH_4^+ in the medium. This happened only at a dilution rate of 0.2 $hr.^{-1}$ or below. Phosphate proved more suitable as a buffer, although its use will prevent a study of phosphate deficiency. Eight of the ten growth deficiencies reported in this project were carried out in Fermentor 1 where the medium of low buffering capacity was used in conjunction with the automatic pH system. This Fermentor was completed many months in advance of Fermentor 2 and several growth limitations were carried out in it before the other was in commission. Reference is duly made in each deficiency experiment to which fermentor was used.

The Criterion of the Dry Weight

Throughout the work described, the basic reference has been to the dry weight of organisms per ml. of culture fluid. While this may not be an ideal reference measurement, there are few conditions reported here where normal cell constituents are grossly diluted by PHB or polysaccharide. The total nitrogen content, or even the total non-dialysable nitrogen, might bear a more direct relation to the amount of protein, and this in turn to the potential enzymic activity, but since the capsule of B. megaterium may have a variable polypeptide component the use of the nitrogen level would seem no better than the dry weight. Further, most continuous culture studies are expressed as dry weight per weight of limiting nutrient and activities, rate or contents, related to the dry weight. Where a component or extracellular product is measured, it is expressed as a percentage of the dry weight and often as a rate of production per mg. dry weight per hr. The rate of production is obtained by multiplying the ratio of the amount of the constituent per mg. dry weight by the dilution rate. Multiplying by the dilution rate instead of the flow rate renders the resulting rate of synthesis of the compound independent of the vessel volume. An

alternative is given, in the case of RNA and DNA, by expressing their content per cell as well. This is particularly important in the case of DNA where the percentage of the dry weight gives little insight into the roles played by the limiting nutriant on DNA synthesis.

Starting Procedure

The starting procedure for each run was similar for all the nutrient limitations. After inoculation and after the organism had established itself, the rate was set at 0.5 $hr.^{-1}$ for 64 hr. (the running in period) and then set at the lowest rate to be tested, usually 0.1 $hr.^{-1}$. The reason for the initial fast rate was that it prevented troublesome foaming conditions which otherwise occured in the first few generations. The reason for starting at the lowest growth rate was as follows. Batch culture experiments of McQuillen (1956) indicated that the doubling time for B. megaterium strain KM was between 60 and 90 min. The shortest doubling time achieved in batch culture in this laboratory was 66 min., equivalent to 0.63 $hr.^{-1}$. However, in the continuous culture vessel D 0.66 $hr.^{-1}$ was the maximum growth rate that could be achieved at the beginning of a run before the organism had been forced

to grow at low D values; by forcing the organism to grow at rates below D 0.2 $hr.^{-1}$, no difficulty was experienced in attaining rates upto, and in excess of 0.8 $hr.^{-1}$, i.e. a doubling time of 52 min.

One experiment was carried out with the complete growth medium (Table 2). Fermentor 1 was run as a repeated dilution vessel, or as a crude type of turbidostat. After the culture had established itself the rate of flow was set so that washout occurred. When the turbidity in the vessel was very low, pumping was slowed and the survivors allowed to grow up, a faster rate being started when the turbidity became appreciable. These cells should, therefore, not have been exposed to any limitation of growth by a deficiency in the medium. This procedure was kept up for 14 days, by which time a rough mutant had outgrown the parent strain. The turbidity was followed during the periods of batch growth, but the doubling time was never below 63 min. (D 0.66 $hr.^{-1}$). The question of selection and adaptation that arises from the experiments is discussed later.

After allowing constant turbidity to prevail for at least 64 hr. at D 0.1 $hr.^{-1}$ and after taking a sample, the

rate was increased by a factor of between 0.1 $hr.^{-1}$ and 0.15 $hr.^{-1}$. In cases where a reduction in rate was required, this was achieved by a reduction in steps of 0.1 - 0.15 $hr.^{-1}$ at 48 hr. intervals until the desired rate was reached.

Table 4

THE EFFECT OF GLUCOSE LIMITATION

Dilution Rate ($hr.^{-1}$)	0.1	0.1	0.2	0.2	0.3	0.4	0.5(x)	0.5	0.6	0.7(1)	0.7(4)	0.8(2)	0.8(3)
Doubling Time (t_d) in hrs.	6.9	6.9	3.44	3.44	2.31	1.73	1.38	1.38	1.15	0.99	0.99	0.87	0.87
Dry Weight (mg/ml.)	0.78	0.74	0.84	0.80	0.87	0.82	0.97	0.90	0.92	0.85	0.81	0.58	0.60
Apparent Yield (mg. dry wt/mg True Yield Carbon)	0.97 (")	0.93 (")	1.05 (")	1.00 (")	1.09 (")	1.02 (")	1.21 (")	1.12 (")	1.15 (")	1.06 (")	1.01 (0.94)	0.73 (0.82)	0.75 (1.05)
Rate of Cell Production (g/l of culture volume/hr.)	0.078	0.074	0.168	0.160	0.261	0.328	0.485	0.450	0.552	0.595	0.567	0.464	0.480
Population Density Total Count/ml x 10^8	-	4.86	8.3	9.6	11.4	6.75	-	7.65	5.9	5.99	-	2.97	-
Mean Cell Mass (ug x 10^{-6})	-	1.52	1.0	0.83	0.76	1.21	-	1.71	1.56	1.42	-	1.9	-
% DNA Content (% of Dry Wt.)	2.9	3.16	3.38	3.27	3.12	3.06	2.26	2.44	2.09	2.15	-	2.2	-
DNA/cell (ug x 10^{-9})	-	48	34	27	24	38	-	29	33	29	-	43	-
% RNA Content (% of Dry Wt.)	13.1	11.8	14.5	14.2	16.6	16.6	-	19.2	21.4	21.8	-	23.6	-
RNA/Cell (ug x 10^{-8})	-	18.0	14.7	11.9	12.7	20.0	-	22.6	33.4	31.0	-	46.2	-
% Nitrogen Content (% of Dry Wt.	12.3	12.4	11.0	-	12.0	12.0	-	10.8	10.5	12.7	-	12.1	-
% Phosphorus Content (% of Dry Wt.)	2.10	1.92	2.02	-	2.20	1.92	1.99	2.34	2.61	1.80	-	2.94	-
Doubling Time (hr.) when suspended in complete medium	3.0	2.17	1.84	-	1.60	1.38	-	1.27	-	1.30	-	-	-

Glucose Limitation

This run, carried out in Fermentor 1, with an ingoing glucose concentration of 2 mg. per ml. was primarily to determine whether under carbon limitation the organism has a capacity to store reserve materials. The results are shown in Tables 4 and 5.

Dry Weight

The pattern of dry weight per ml., as with all other cellular and extracellular constituents, is recorded against the dilution rate. The dry weight was at a maximum at D 0.5 - 0.6 $hr.^{-1}$, quickly dropping at higher growth rates but dropping gradually at lower rates as the bacteria became less efficient in converting glucose to cell material. A possible explanation is that at the low glucose concentrations prevailing, the energy of maintenance becomes proportionally greater.

Yield

There are several possible methods of reporting yield. Undoubtedly it would have been best to recover all the ingoing carbon in the bacteria and the effluent medium as well as carbon dioxide evolved, but facilities were not available for these measurements. An alternative is to record the ratio of dry weight to carbon input (Apparent Yield). However, a discrepancy occurs at high dilution

Table 5

THE EFFECT OF GLUCOSE LIMITATION (Cont.)

Dilution Rate ($hr.^{-1}$)	0.1	0.1	0.2	0.2	0.3	0.4	0.5(a)	0.5	0.6	0.7(1)	0.7(4)	0.8(2)	0.8(3)
% Polysaccharide Content (% of Dry Wt.)	7.8	6.4	6.4	-	7.7	8.5	9.8	7.2	8.7	9.4	-	11.4	9.4
% Glycogen Content (% of Dry Wt.)	1.6	1.1	1.4	-	1.1	0.9	1.4	1.5	1.2	1.2	-	1.3	-
% PHB Content (% of Dry Wt.)	0	0	0	0	5.5	12.0	10.4	6.3	7.7	4.9	7.2	3.0	5.4
Rate of PHB Production (µg/mg dry wt/hr.)	0	0	0	0	16.5	48.0	52.0	31.2	46.2	34.4	50.5	24.0	42.8
Rate of Depolymerisation in Washed Cell Suspensions (µg/mg dry wt/hr.)					18	-	-	20	-	13	-	-	-
Glucose Yield Constant. (mg. dry wt./mg. Glucose)	0.40	0.37	0.42	0.40	0.44	0.41	0.49	0.45	0.46	0.43	0.43	0.33	0.42
Glucose Oxidation Rate (mg glucose/mg dry wt/hr.)	0.25	0.27	0.48	0.50	0.69	0.98	1.03	1.11	1.30	1.65	1.65	2.42	1.90
Residual glucose in culture medium (µg/ml.)	2	-	2	-	2	2	-	2	2	2	88	224	582
% Pyruvic Acid (% of Dry Wt.)	-	-	-	-	0	0	-	0	0	0	6.35	2.2	4.25
Rate of Pyruvate Production (µg/mg. dry wt/hr.)	-	-	-	-	0	0	-	0	0	0	30.6	17.5	34.0
% Oxoglutaric Acid (% of Dry Wt.)	-	-	-	-	0	0	-	0	0	0	2.5	7.6	4
Rate of Oxoglutarate Production (µg/mg. dry wt/hr.)	-	-	-	-	0	0	-	0	0	0	18	61	32
% Acetic Acid (% of Dry Wt.)	-	-	-	-	0	Low	Low	2.7	8.5	20.4	36.6	60.2	25.0
Rate of Acetate Production (µg/mg. dry wt/hr.)	-	-	-	-	-	Low	Low	13.5	51.0	143.0	256.0	480.0	280.0

rates where all the glucose is not utilised and another figure for yield can be obtained (True Yield) by expressing the ratio of dry weight to glucose carbon utilised. Both Yield values are nearly identical except for the last three columns of Table 4.

The Apparent Yield mirrors the dry weight pattern at all growth rates. The True Yield does so only at the lower growth rates, not at D 0.7 and D 0.8hr.$^{-1}$. It might be argued that the True Yield constant is unnecessary because an extracellular concentration of glucose is required to produce high growth rates, suggesting that a permease system required a high external concentration to drive glucose into the cell at the required rate. If this argument is correct the True Yield constant is misleading, but if some synthetic system in the cell such as a step in the synthesis of one particular amino acid not directly related to glucose entry is involved, then the term True Yield is correct. These experiments do not suggest which is correct.

Selection Pressures

The four highest D values recorded were carried out in the order mentioned in brackets in the D value

column. After D 0.8 hr.$^{-1}$ (2) had been carried out, a flow rate of D 0.9 hr.$^{-1}$ was set and near washout ensued after 36 hr., a low constant turbidity indicated there were too few organisms to be of practical use. The rate was then set at D 0.8 hr.$^{-1}$ (3) and finally at D 0.7 hr.$^{-1}$. The value marked D 0.5 hr.$^{-1}$ (X) was a sample taken during the 'running in' period. The duplication of results from similar dilution rates gave significantly different values not only in glucose deficiency, but in other runs as well. The differencies obtained were much greater than the degree of accuracy of most of the determinations, indicating that the changes were inherent in the bacillus itself.

Measurement of Doubling Times in Batch Culture

A method of showing that different genotypes develop at different dilution rates is to measure the doubling time of organisms from different dilution rates on transfer to batch culture. Semi-log plots of turbidity against time gave a straight line after 1 - 2 hr. indicating that the cells were not adapting from their respective growth rates in the vessel. The different growth rates found (Table 4) were always faster than in the vessel, but none the less

Mean generation time

210 min.

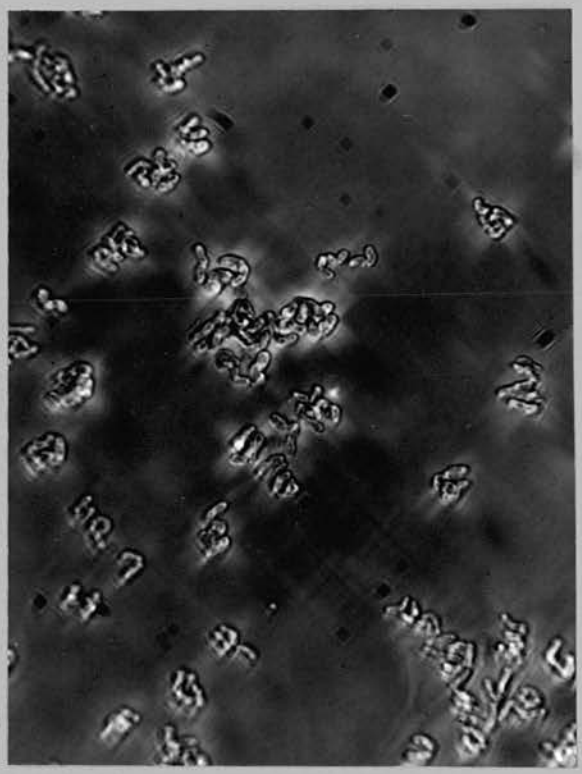


Fig. 28a

52 min.

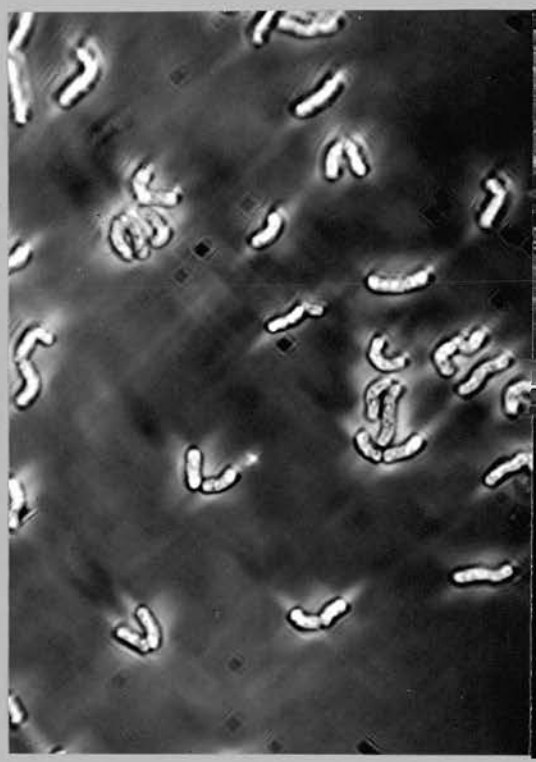


Fig. 28b

Photomicrographs of Bacillus megaterium grown in continuous cu-
at different growth rates. Carbon limitation, water mount, medi-
dark phase contrast x 1000.

indicate that genetic selection is taking place.

Changes in colony type of Population

Rough mutants were identified on synthetic and nutrient agar plates during the whole of this run except at D 0.8 hr.$^{-1}$. However, they never exceeded 10% of the total on any one plate. Similar rough mutants were detected during all the other growth limitation experiments although few, if any, ever occured at D 0.8 hr.$^{-1}$.

Sporing colonies were only noted at intermediate growth rates in sulphur deficiency, only a few ever being detected. Smooth, non-sporing colonies were detected in moderate amounts in potassium deficiency from D 0.1 - 0.35 hr.$^{-1}$. No further mention is made of these colony forms in the results section.

Population Density and Mean (Dry) Cell Mass

The population density (total count) was measured to give some idea of the individual dry mass of the cells grown at different D values under the different growth limitations. Figs. 28 a & b show photomicrographs of cells from D values 0.2 hr.$^{-1}$ and D 0.8 hr.$^{-1}$. Table 4 shows that the mean cell mass is at a minimum at D 0.3hr.$^{-1}$, indicating that division is retarded at values below this point. An increase in cell mass and cell size above

D 0.3 hr.$^{-1}$ agrees well with other results for carbon deficiency (Herbert, 1958).

Nucleic Acids

The inhibition of division noted in the last section below D 0.3 hr.$^{-1}$ is not because of a deficiency of DNA. Table 4 shows that there is almost twice as much DNA per cell at D 0.1 hr.$^{-1}$ as at D 0.3 hr.$^{-1}$. The only correspondingly high amount of DNA per cell occurs at the fastest rate recorded (D 0.8 hr.$^{-1}$). Assuming that the lowest values of DNA recorded for glucose and nitrogen deficiency (14 -16 ug x10^{-9}) represent one nucleus per cell, then the cells at D 0.8 hr.$^{-1}$ must be approximately trinucleate. This calculation is used in later runs to describe the number of nuclei per cell.

The percentage of RNA shows a gradual rise with increasing growth rate, the content per cell reflecting the influence of the growth rate rather than the DNA pattern. The absolute values for the percentage of RNA appear to be about 6 - 9% higher than those reported elsewhere for B. megaterium at the same growth rates (Herbert, 1958) although the pattern and rate of increase is similar. Corresponding high values for RNA were obtained for all limitations. Herbert does not record his method for RNA

determination but that employed here used the factor of 4.91 of Schaechter et al. (1958) for converting ribose to RNA. The factor implies that only the purine ribose is reacting and it is possible that there is some reaction from the pyrimidine component in our cells. However, Herbert's figures for Aerobacter aerogenes are low by 3% compared with those of Postgate and Hunter (1962) for the same organisms under similar carbon limitation.

Total Nitrogen

It was decided at the outset of the project to follow the percentage of nitrogen in the dry weight to see whether under any limitation there was an indication that dilution of protein material occured. Only in nitrogen limitation was there a drop in the value, a fact which can possibly be explained by a reduction in the cell wall contents.

The percentage of nitrogen found (see Table 4) remains much the same at all dilution rates. A possible source of error in the method not previously mentioned is that the cells used in the determination had been washed three times in 1% (W/V) NaCl and undoubtedly some nitrogenous material would be lost. The fact that RNA and associated protein material increases more than two-fold overall and yet leaves the total nitrogen content of the

dry weight much as before indicates that the small expected drop is compensated for by an increase in another nitrogen containing component.

Total Polysaccharide and Polyglucose

No definite picture emerged from the total polysaccharide content. Duplicate dilution rates gave widely different values, but by averaging the results of similar dilution rates a slight increase in polysaccharide content was noted at higher growth rates.

The polyglucose level was approximately 1 - 1.5 % of the dry weight at all rates of growth. This low level of polyglucose corresponds to a structural component of polyglucose like material found by Ghuysen (1961) to occur in the cell walls of B. megaterium strain KM. The significance of this structural component is discussed later in relation to intracellular polyglucose storage.

PHB The PHB content and its rate of synthesis reached a maximum at D 0.4 - 0.5 $hr.^{-1}$, dropping slightly at faster rates of growth. At the two lowest dilution rates there was less than 0.5% PHB although very small sudanophilic granules still occurred and it is conceivable that the granules were composed of neutral lipid rather than PHB. The rate of PHB breakdown was also recorded

in washed cell suspensions for some of the dilution rates. The results given in Table 5 show that the two lower growth rates gave nearly identical depolymerisation rates while the faster growth rate gave a reduced depolymerisation rate. It was impossible to determine the rate of depolymerisation at D 0.8 $hr.^{-1}$ since the cells autolysed within 30 min of transfer to the suspending medium. Good agreement was obtained by comparing the PHB content, determined by the method of Law & Slepecky (1961) with the initial turbidity at the start of a degradation experiment.

Glucose Yield Constant and the Glucose Oxidation Rate

The glucose yield constant (GYC) is the ratio of the dry weight per unit of glucose oxidised. It has the same meaning as the True Yield constant in this run as glucose is the limiting factor, but in other runs the Yield Constant relates to Nitrogen, Sulphur, etc. As glucose was used in nearly all other studies, the Glucose Oxidation Rate (GOR) is a useful reference to determine the relationship between the growth rate and the oxidation of glucose. The GOR is the amount of glucose oxidised per unit of dry weight per hr. and as the glucose was nearly all utilised, the

GOR shows a step-wise increase at each growth rate.

The GOR also showed at which point maximum oxygen demand occured. To calculate the oxygen uptake rate the equations given by Pirt (1957) for oxygen demand in a continuous culture should show if there is a shortage of dissolved oxygen to limit growth. The rate of oxygen uptake by growing cells is given by:

$$- \frac{dc}{dt} \simeq P\mu (So - S)$$

where C = conc. of dissolved oxygen

P = ratio of 1 mole of growth-limiting substrate utilised to the number of moles of O_2 required to oxidise this substrate.

So = Conc. of limiting carbon in the ingoing medium = 66.7 m.M.

S = Conc. of limiting carbon in the vessel.

$\Delta S = So - S$

$\mu = D$

In Glucose limitation $\Delta S \simeq So$.

$$\therefore \quad - \frac{dc}{dt} \simeq PDSo$$

Because CO_2 was not measured in the effluent gas and the cell content of carbon was not measured either, two assumptions are made:

1. The difference between the carbon in the cells

plus the carbon in the acetic acid in the medium and the ingoing glucose carbon was produced as CO_2. Cell carbon is assumed to be at the same state of reduction as glucose.

2. The bacteria contain 50% of their dry weight as carbon.

Using D 0.7 $hr.^{-1}$ as an example and averaging the two dry weights, then 0.83 mg. of cells contain 0.415 mg. carbon. 28% of 0.83 mg. is produced as acetic acid i.e. 0.232 mg. which contains 0.093 mg. carbon.

Then the total carbon recovered was 0.415 + 0.093 = 0.508 mg. 0.508 mg. carbon per ml. represents 42.4 m.M. of carbon per l.

Then the amount of glucose oxidised to CO_2 was

66.7 - 42.4 = 24.3 m.M. of carbon or oxygen

$$\therefore \quad P = \frac{24.3}{66.6} = 0.36$$

$$\therefore \quad -\frac{dc}{dt} \simeq 0.36 \times 07 \times 66.7$$

16.8 m.M. of O_2/l/hr.

The sulphite oxidation rate found for the conditions of stirring and aeration used was nearly 300 m.M. of O_2/l/hr.

Therefore, oxygen exceeded the demand by twenty times.

Gram Staining Properties

Gram staining of the cells was carried out as routine at each dilution rate. Strain KM of B. megaterium is variable in reaction. The results showed that cells grown below D 0.3 $hr.^{-1}$ were Gram-negative and above D 0.5 $hr.^{-1}$, were almost entirely Gram-positive. The cells grown between and including these rates were a mixture of Gram-positive and Gram-negative, one always predominating. Similar findings were recorded for all other limitations.

Generally the literature suggests that in Gram-variable organisms, most cells in the log phase are Gram-positive whereas stationary phase cultures are nearly always Gram-negative. It is most unlikely that the observed changes in our culture are due to selection as it is now generally agreed that the Gram reaction is not an 'all or none' reaction. Salton (1962) suggests that the proportions, arrangement and total amounts of the components of the cell walls of bacteria quantitatively affect the Gram reaction. He further suggests that the thickness of the mucopeptide polymer in Gram positive organisms renders the crystal violet - iodine complex

impermeable to washing with alcohol. Gram-variable properties are, therefore, explicable on the basis of the degree of thickness of the mucopeptide layers. Our experiments do indicate that the growth rate is implicated, if only indirectly, with the degree of Gram-positive staining. In the light of Salton's suggestions, it is not inconceivable that the thickness of the mucopeptide layer, its porosity, or the physiochemical nature of the cell wall is affected by the growth rate.

Extracellular Products

The strain of B. megaterium used in these experiments has been shown to produce acetic acid, pyruvic acid and 2-oxoglutaric acid in varying amounts according to the limiting nutrient when glucose was used as the carbon source. Other products were tested for but found to be absent, namely lactic acid, formic acid, oxalacetic acid and acetoacetic acid.

Table 5 shows that very small amounts of acetic acid occurred at D 0.5 $hr.^{-1}$ and possibly even below at D 0.4 $hr.^{-1}$, the rate of production steadily increasing with increasing growth rate. Acetic acid production in glucose limitation is very similar to the results of Pirt (1957) who cultured Aerobacter cloacae continuously under

very similar conditions to our own. The production of acetic acid during glucose deficiency at D 0.4 - 0.5 $hr.^{-1}$ and at faster growth rates may mean that the combined rates of breakdown of glucose by the Embden-Meyerhof pathway and the Hexose-Monophosphate shunt produce more acetyl fragments than subsequent systems can oxidise or build into cell material. This result is discussed later in relation to the findings from subsequent runs. The production of the two oxoacids only occurred at the highest growth rates where there was a large amount of glucose in excess in the culture supernate, suggesting that some internal factor was limiting cell yield.

Total Phosphorus Content and Volutin Staining

Total phosphorus content and volutin stainability were carried out at each dilution rate. It was hoped that if volutin staining occurred, the total phosphorus content would also reflect storage of polyphosphate. As there is a high content of phosphorus in nucleic acids (11%) the continued rise in percent RNA with increasing growth rates should be reflected in the total phosphorus content. This is apparent from growth rates of 0.5 $hr.^{-1}$ and upwards, whereas below this, the phosphorus content was fairly constant. Volutin staining was recorded at D 0.1 $hr.^{-1}$ up to

D 0.5 hr.$^{-1}$. There were seldom more than one or two granules per cell but in view of the fact that volutin staining occurred in all deficiencies at lower growth rates it is dubious what this staining represents.

Extracellular Depolymerisation Studies

Extracellular depolymerisation studies were carried out using carbon-deficient cells growing at D 0.3 hr.$^{-1}$. Pumping was switched off and the cells allowed to degrade their PHB over a period of 2.5 hr. This time was known to be sufficient to ensure that all PHB could be degraded. Degradation was allowed because the depolymerase is known to be absorbed on the surface of the granules, (Wilkinson, unpublished results) and removing the polymer ensures that the enzyme is in the soluble form from disrupted cells. After removal from the vessel these cells (300 ml.) were centrifuged, suspended in 0.05 M Tris-HCl buffer pH 8 and subjected to sonic oscillation in ice water for 12 mins. The resulting material was centrifuged at 20,000G to remove all particulate matter and then the supernate was made up to 30 ml. with Tris buffer and stored at 0°.

Native polymer granules were extracted from cells grown under nitrogen deficiency in Fermentor 2 at D 0.3hr.$^{-1}$

by the method of Merrick and Doudoroff (1961). This involved centrifuging cells (300 ml.) at a dry weight of 0.78 mg. per ml. and suspending them in 0.05 M. Tris buffer pH 8 (30 ml.), subsequently they were treated with lysozyme (0.3 %Wt/Wt. of cells) for 30 mins. and cell-free extracts prepared by sonic ascillation of the protoplasts for 2 mins.; this latter process also frees the polymer particlesffrom cell membranes. The particles were concentrated by centrifugation at 800 g. for 3 mins. and washed three times in 0.05 M. Tris buffer pH 8 before suspending to a final volume of 30 ml. They were stored at 0°.

The following experiment, shown in Table 6, was carried out using the two extracts and the fall in turbidity measured at 530 mμ.

Table 6

Depolymerisation of PHB Granules in extracellular extracts of Bacillus Megaterium

Preparation	Turbidity Values						
	0	2hr.	4hr.	6hr.	9hr.	12hr.	24hr.
1.5 ml. of Granules + 8.5 ml. of 0.05 M Tris	0.505	.510	.053	.505	.500	.495	.491
	0.510	.513	.511	.508	.508	.510	.508
1.5 ml. of Granules + 7 ml. of 0.05 M Tris + 1.5 ml. of Soluble Depolymerase	0.508	.433	.351	.302	.275	.245	.240
	0.520	.450	.382	.330	.299	.285	.240

The rate of drop of turbidity was only half that recorded for intact cells depleting their PHB reserves, whether from carbon or nitrogen deficiency. The experiment was repeated with fresh granules and soluble enzyme preparation but no greater rate of breakdown was achieved. Cells from D 0.35 $hr.^{-1}$ grown under nitrogen limitation are capable of breaking down their own PHB. Therefore, the method of preparation employed here must result in the destruction or the loss of the depolymerase on these native granules. The preparation containing the soluble depolymerase retains at least part of its activity as is proved by the experiment recorded in Table 6. Even if this soluble enzyme is partially destroyed, these experiments did not show a faster rate of depolymerisation <u>in vitro</u>.

An interesting point arose during the period when the cells from glucose deficiency at D 0.3 $hr.^{-1}$ were degrading their PHB reserves after pumping had been stopped. During this run, a very low rate of alkali addition was required. Approximately 3 min. after stopping the pumping of fresh medium, regular addition of acid was required to keep the pH at 7.3. The rate of acid addition slowed down after 15 - 20 min. and became progressively slower during the 2.5 hr. The most probably explanation

of the base production is that in addition to breakdown of PHB, there was a simultaneous degradation of RNA, or protein (amino acid pool), or both, resulting in deamination and production of NH_4^+ ions into the medium.

Table 7

THE EFFECT OF GLUCOSE LIMITATION + 0.1 M SODIUM ACETATE

Dilution Rate ($hr.^{-1}$)	0.1	0.2	0.35	0.5	0.54	0.60	0.65
Doubling Time (td) in hrs.	6.9	3.44	1.97	1.38	1.28	1.15	1.06
Dry Weight (ug /ml.)	1.24	1.61	1.46	1.11	1.16	1.07	0.79
Yield (mg. dry wt/mg. glucose carbon)	1.55	2.01	1.82	1.39	1.45	1.34	0.99
Rate of Cell Production (g./l. of culture vessel/hr.	0.12	0.32	0.51	0.56	0.63	0.64	0.54
Population Density Total Count/ml. x 10^8	2.94	3.68	4.15	6.8	-	5.01	2.3
Mean Cell Mass (ug x 10^{-6})	4.0	4.4	3.5	1.6	-	2.1	3.4
% DNA Content (% of Dry Wt.)	1.31	1.42	1.50	2.18	-	2.26	1.95
DNA/cell (ug x 10^{-9})	55.0	62.0	52.5	35.2	-	40.3	67.0
% RNA Content (% of Dry Wt.)	5.4	5.8	10.4	16.2	-	17.0	21.8
RNA/cell (ug x 10^{-8})	22.8	25.4	36.5	26.5	-	36.0	75.0
% Nitrogen Content (% of Dry Wt.)	11.5	11.7	12.3	11.9	-	10.3	10.9
% Polysaccharide Content (% of Dry Wt.)	3.3	2.9	4.3	6.3	-	7.5	6.2
% PHB Content (% of Dry Wt.)	0	0	0	2.3	4.7	7.5	11.3
Rate of PHB Production (ug/mg dry wt/hr.)	0	0	0	11.5	25.0	45.0	73.5
% Phosphorus Content (% of Dry Wt)	3.51	2.72	2.90	2.34	-	2.50	2.50

Glucose Limitation in the presence of excess acetate (0.1M)

This run was carried out in order to clarify the picture in relation to the results of Macrae and Wilkinson (1958a) who found that in a glucose limiting batch culture plus excess acetate, cells harvested in the stationary phase had an abnormally high content of PHB. Their experiment followed from washed-cell studies (Macrae & Wilkinson, (1958b), where it was found that glucose, and especially pyruvate, gave a high rate of PHB synthesis, but the addition of acetate to glucose or pyruvate gave an even higher rate of synthesis. The results of this experiment, which was carried out in Fermentor 1, are shown in Table 7.

Dry Weight, Yield

Although B. megaterium does not grow on acetate, presumably because it has no glyoxalate pathway, the figures for the dry weight show that acetate can be used. A comparison of these dry weights and the corresponding figures for glycose are shown in Table 8.

Table 8

Comparison of Dry Weight per ml. of Glucose and Glucose plus excess Acetate Deficiencies

Results are shown as mg/ml.

Deficiency	Dilution Rate ($hr.^{-1}$)				
	0.1	0.2	0.35	0.5	0.6
Glucose plus excess acetate Deficiency	1.24	1.61	1.46	1.11	1.07
Glucose Deficiency	0.76	0.82	0.885	0.935	0.92
Difference	0.48	0.79	0.575	0.175	0.15

Therefore, at D 0.2 $hr.^{-1}$, there is almost 100% addition to the dry weight, perhaps suggestive of 3 mole. of acetate per mole of glucose. Acetate incorporation would then be dependant on acetyl phosphate or acetyl CoA, or the energy requirement to produce these intermediates.

This was the only limitation with glucose as energy source, where a dilution rate of 0.8 $hr.^{-1}$ was unobtainable after several attempts at D 0.7 $hr.^{-1}$. Washout occurred between D 0.65 - 0.7 $hr.^{-1}$. The reduced maximum growth rate (similar to that of the parent strain) is most probably due to the effect of the high concentration of acetate either preventing the selection which normally occurs at low growth rates or acting specificially as a growth inhibitor. Studies by Collins and Kornberg (1960) and Kornberg, Phizackerly and Sadler (1960), have shown

that acetate ions inhibit the growth rate of E. coli by effectively lowering the level of TCA cycle intermediates.

Population Density and Mean Cell Mass

In this run the cells were unusually large and ovoid at low growth rates, looking more like yeasts than bacilli. Indeed, the population density was so low that at D 0.1 - 0.35 $hr.^{-1}$ the mean cell mass was three times the weight of glucose-grown cells at the corresponding D values. Even at faster growth rates, the cells from this run were larger than the corresponding glucose-grown cells, as though there was an inhibition of cell division.

Nucleic Acids

The increase in cell size was not due to lack of DNA as the cells are probably trinucleate at low D values although, in relation to total cell contents, the percentage DNA of the dry weight is low. The percentage content of RNA was also low at the lowest growth rates but the content per cell was somewhat higher than in glucose deficiency. At the highest growth rates both percentage RNA and RNA per cell were very much higher than glucose-deficient grown cells, suggesting again that division was inhibited.

Total Polysaccharide

Polysaccharide levels were very low between D 0.1 - 0.35 $hr.^{-1}$. A possible explanation may be that as

the size, and therefore, the weight of an individual cell increases, the surface area per weight ratio decreases giving a smaller amount of cell wall per unit mass. Calculation of the amount of polysaccharide per cell shows there is approximately twice as much as in the corresponding glucose grown cells at low growth rates.

PHB

In the cells from D 0.1 - 0.35 $hr.^{-1}$ the cytoplasm stained uniformly with Sudan Black indicating that the entire cytoplasm or cytoplasmic membrane was composed, at least partly, of lipid. The cell wall was not visibly stained. No individual granules could be seen through this black stain although chemical determinations of PHB proved that there was none present during these three dilution rates. Normal staining occurred at D 0.5 $hr.^{-1}$ and at faster rates of growth. Some difficulty was experienced in the alkaline hypochlorite treatment of these cells from low growth rates. There remained a brownish-buff residue in place of the usual white suspension of granules. This brown material tended to settle on the bottom of the tube rather than remain as a suspension in the manner of PHB granules. An aqueous suspension of the cells was also a much darker brown than the corresponding glucose-limited cells from similar

low growth rates. However, the dark colour disappeared in cells from D 0.5 $hr.^{-1}$, where there was much less incorporation of acetate, and as shown in Table 7, PHB occured in increasing amounts at faster growth rates.

A preliminary investigation was carried out on this brown material remaining after hypochlorite treatment of the cells. It was insoluble in chloroform showing conclusively that it was not PHB. It was hydrolysed by heating for 24 hr. with 6N HCl. in a sealed tube to give a yellow solution. No sugar-/or nitrogen-containing compounds were identified in the hydrolysate using conventional chromatographic methods.

The above results show that the assimilated acetate was not incorporated as polysaccharide or PHB. Some acetate must form conventional lipid as was shown by the Sudan Black stain. However, this lipid cannot be formed in excessive amounts as the total nitrogen content is similar to that of cells grown in glucose deficiency. The lower contents of RNA, DNA and polysaccharide may be due to a replacement by lipid, thus accounting for the constant nitrogen level. To explain the large increase in dry weight and the constant nitrogen level, acetate is presumably synthesised mainly into protein components.

Unfortunately there is no evidence to show that the rate of carbon dioxide production was any faster than in glucose deficiency. Some acetate will be oxidised, but the amount of oxidation in relation to incorporation is the important factor and this is still unsolved. Another feature is that if acetate assimilation was a slow process, then we would expect to find at least a similar or higher dry weight at D 0.1 $hr.^{-1}$ as at D 0.2 $hr.^{-1}$, but in fact it was lower. This low dry weight indicates that the glucose metabolism probably affects acetate uptake by supplying condensing intermediates and energy. Indeed, if it were proved that the rate of acetate oxidation was low during times when large amounts were incorporated, the energy being provided by glucose metabolism, the resultant low energy levels in the cell would account for inhibition of division; acetate assimilation during glucose metabolism would then be an example of carbon excess and energy deficiency.;

Phosphorus and Volutin Staining

The only other material determined in the run and not mentioned so far is phosphorus. Volutin staining was quite negative but total phosphorus was high at the lower growth rates. In view of this, it was decided to carry out a fractionation procedure to determine the distribution of phosphorus compounds. The analyses were performed on cells washed

twice in distilled water, the results of which are shown in Table 9.

Table 9

Fractionation of Cells from Glucose Deficiency in the presence of excess acetate by the method of Juni et al. (1953) Results are shown as mg. per 100 mg. of cells.

Fraction	Probable Nature	Dilution Rate		
		0.2	0.35	0.65
S_2	Free nucleotide + Acid soluble organic Phosphate	0.79	0.86	0.32
S_3	Inorganic Orthophosphate	0.12	0.16	.09
R_3	Inorganic Polyphosphate	0.06	0.07	0.02
S_4	Phospholipid	0.05	0.03	0.03
S_5	Nucleic Acids, Pyrophosphate, Phosphoprotein	1.20	1.25	1.36
R_8	Unknown. 7 min. hydrolysable Phosphate	0.10	0.15	0.15
R_T	Phosphorus in residue	0.04	0.06	0.08
S_8	7 min. hydrolysable Phosphate + Polyphosphate	0.02	0.13	0.05
	Total	2.38	2.71	2.10
	% Recovery	87.5	94.5	90.0

It is apparent that the TCA soluble S_2 fraction accounts for much of the increased phosphate content at D 0.2 and D 0.35 hr.$^{-1}$. The fraction S_5 contains much more phosphorus than the nucleic acid determinations allow.

It is also apparent that the level of polyphosphate was small in all cases; indeed the amount was insufficient to characterise by precipitation at pH 2.5 with barium. In view of this small amount of possible polyphosphate these experiments were discontinued.

Carbon Limitations other than Glucose

The object of this run was to determine if glucose deficiency was unique in allowing PHB formation and generally to see if the yield and dry weight patterns were similar. A departure from the usual technique was that the parent strain was not used at the start of each deficiency, or in other words, when one deficiency experiment was completed, another nutrient was pumped in immediately. Deficiencies of succinate, lactate or glycerol as sole carbon and energy sources were carried out in Fermentor 1.

In order to compare the theoretical yield of dry weight which should be given by each deficiency it is necessary to calculate the amount of energy that will be derived from the oxidation of a molecule of substrate to carbon dioxide and water. It will be remembered that the concentration of each carbon source was such that there was an equivalent amount of carbon molecules per ml. in each carbon limitation. The equations which follow are arranged so that the total carbon of each substrate is the same and direct comparison of the number of oxygen atoms required to convert the substrate to carbon dioxide

and water can be made. The Yield (Y), unit dry weight per unit of substrate required, should be proportional to the oxygen requirement.

Glucose ($C_6H_{12}O_6$)

$$C_6H_{12} + O_6 + 12\ O = 6\ CO_2 + 6\ H_2O$$

Succinate ($C_4H_6O_4$) 1.5 mole.

$$C_6H_9O_6 + 10.5\ O = 6\ CO_2 + 4.5\ H_2O$$

$$\frac{Yo_2 \text{ succinate}}{Yo_2 \text{ glucose}} \qquad \frac{10.5}{12} = 0.875$$

Lactate ($C_3H_6O_3$) 2 mole

$$C_6H_{12}O_6 + 12O = 6\ CO_2 + 6\ H_2O$$

$$\frac{Yo_2 \text{ lactate}}{Yo_2 \text{ glucose}} = \frac{12}{12} = 1$$

Glycerol ($C_3H_8O_3$) 2 mole

$$C_6H_{16}O_6 + 14O = 6\ CO_2 + 8\ H_2O$$

$$\frac{Yo_2 \text{ glycerol}}{Yo_2 \text{ glucose}} = \frac{14}{12} = 1.17$$

Therefore, glycerol limitation should give the highest yields, lactate should be similar to glucose and succinate should be the lowest. Comparison of the dry weights of each deficiency experiment with dry weight at equivalent D values in the glucose limitation run is shown

TABLE 10

THE EFFECT OF DIFFERENT CARBON LIMITATIONS

Limiting Nutrient	Dilution Rate ($hr.^{-1}$)	Doubling Time (hr.)	Dry Weight (mg/ml.)	Yield (mg. dry weight/ mg. carbon).	Yield Ratio (Y/ Y Glucose)	% Content of PHB	Rate of PHB Production	% Content of Polysaccharide
Succinate	0.1	6.9	0.57	0.71	0.75	0.0	-	3.8
	0.2	3.44	0.63	0.79	0.77	0.0	-	3.8
	0.3	2.31	0.65	0.81	0.74	0.9	2.7	3.4
	0.4	1.73	0.70	0.88	0.86	4.4	17.6	3.5
	0.48	1.44	0.68	0.85	-	5.6	26.6	3.8
Lactate	0.1	6.9	0.67	0.84	0.89	0.0	-	3.8
	0.2	3.44	0.75	0.94	0.92	0.5	1.0	3.9
	0.3	2.31	0.78	0.96	0.88	7.0	21.0	-
	0.34	2.04	0.74	0.92	-	10.2	34.8	4.2
Glycerol	0.1	6.9	1.09	1.36	1.43	0.0	-	8.3
	0.2	3.44	1.00	1.25	1.22	0.0	-	6.4
	0.3	2.31	0.92	1.15	1.05	6.9	20.7	-
	0.4	1.73	0.81	1.01	.99	14.4	57.6	8.3
	0.46	1.51	0.72	0.90	-	14.0	64.4	9.2

in the ratio of dry weights in Table 10.

Succinate Limitation

The dry weights follow the same pattern as glucose up to D 0.4 $hr.^{-1}$, washout occurring at just above D 0.48 $hr.^{-1}$. The Yield of dry weight was lower than expected by the theory as outlined above. However, the ratio of Succinate Yield constant (SYC) to GYC is nearly constant at all D values. A possible explanation is that in a purely synthetic medium of succinate and inorganic salts, a finite amount of energy will be required to synthesise molecules such as ribose and 2-deoxyribose for nucleic acid synthesis, and glucose and carbohydrates which occur in the cell wall; the yield of cells might, therefore, be expected to be reduced. The low polysaccharide content of the cells supports this latter contention.

The most interesting point of the run, however, is that PHB production only occurred above a dilution rate of D 0.3 $^{-1}$, the same value as glucose deficiency. The values of PHB content were all much lower, but nevertheless it did occur in increasing amount with increasing growth rate.

Lactate Limitation

Dry weight in lactate limitation follows a similar

pattern to glucose and succinate limitation. Yields of dry weight were again reduced in relation to glucose limitation, but were not as low as in succinate limitation. The ratio of Lactose to Glucose Yield Constants is similar at each dilution rate but the value is lower than that calculated, probably reflecting the fact that carbohydrate production required a finite amount of energy which in cells grown under glucose limitation was available for production of new cell material. Polysaccharide production was again low which lends support to this idea.

This run was, however, characterised by the slow maximum specific growth rate which was slower than in the succinate experiment. Why succinate should give a faster maximum rate of growth is unknown.

PHB again appeared at a dilution rate corresponding to that of other carbon and energy sources. Its high rate of synthesis at the highest growth rates suggests that the oxidation of lactate to pyruvate is unlikely to be the cause of the long doubling time.

Glycerol Limitation

The dry weight pattern was quite the reverse of that found in the other carbon limitations. Neither were

the results of Herbert (1958) and of Postgate and Hunter (1962) for glycerol limitation with A. aerogenes similar; their dry weight patterns resemble the previous runs with succinate, lactate, and glucose and their reported maximum growth rate in glycerol limitation was also much faster. The ratio of Glycerol to Glucose Yield Constants was not similar at different dilution rates. The theoretical figure of 1.17 was exceeded at D 0.1 and 0.2 $hr.^{-1}$, but all faster rates were belowtthis figure.

It is unlikely that the rate of entry of glycerol was the rate-limiting step causing the early washout in glycerol limitation, because PHB accumulates to a great extent; this indicates that some intermediates, at least, are not in short supply. The presence of these energy rich intermediates suggests that energy was not in short supply either. The shape of the dry weight curve, which resembles that in glucose deficiency from D 0.6 - 0.8 $hr.^{-1}$, may indicate that glycerol and derived intermediates were accumulating in the culture medium, although this latter point was not investigated. Assuming these three points are correct, therefore, there exists in the cell an excess of glycerol, some intermediates, and energy. Unless growth was specifically inhibited by glycerol or one of the

intermediates between glycerol and triose phosphate, it seems likely that growth was slowed down because of the lack of some key substance produced by an intermediary process. This key substance might well be in the production of glucose or ribose but for the fact that the polysaccharide content should be as low as succinate or lactate, but is as high as glucose deficiency. If there was a growth rate limiting step between triose phosphate and carbohydrate, the high polysaccharide content in glycerol deficiency argues against the proposition and suggests that in each type of carbon deficiency a different mechanism controls the maximum growth rate.

The shape of the growth curve demands some explanation as it suggests that at low D values, where yields are higher than in other carbon limitations, the cell has a very efficient system of converting glycerol to cell material; glycerol-limited cells, just as in other carbon deficiencies will have an energy of maintenance requirement. The increased dry weight at low D values might reflect an ability to couple oxidation of reduced components to fixation of carbon dioxide. Another possibility is that if different genotypes are selected in other carbon limitations at low

D values, then this process of selection of strains which give poor utilisation of the carbon substrate may be inhibited by glycerol, or one of its derivatives.

Table 11

The Effect of Nitrogen Deficiency

Dilution Rate ($hr.^{-1}$)	0.085	0.2	0.2	0.35	0.5	0.65	0.80
Doubling time (hr.)	8.1	3.44	3.44	1.97	1.38	1.06	0.87
Dry Weight (mg./ml.)	1.94 (2.04)	1.98	2.05	2.01	1.85	1.70	1.55
Yield (mg. dry wt./mg. N.)	10.2 (11.3)	10.4	10.8	10.6	9.7	8.9	8.2
Rate of Cell Production (g./l./hr.)	0.17	0.40	0.41	0.70	0.94	1.11	1.24
Population Density (Total Count/ml. x 10^{-9})	2.21	2.29	2.77	2.09	1.25	0.774	0.55
Mean Cell Mass (ug x 10^{-6})	0.88	0.87	0.74	0.96	1.48	2.20	2.81
% DNA Content (% of Dry Wt.)	2.14	1.89	1.93	1.79	1.47	1.71	1.81
DNA/cell (ug x 10^{-9})	18.8	16.4	14.3	17.3	21.8	37.8	51.4
% RNA Content (% of Dry Wt.)	8.0	10.2	10.8	12.6	13.6	18.0	21.5
RNA/cell (ug x 10^{-8})	7.2	8.9	8.0	12.1	23.2	39.6	60.5
% Nitrogen Content (% of Dry Wt.)	8.7	9.4	9.0	9.1	9.9	10.5	10.3

Nitrogen Limitation

This run was carried out to confirm that under nitrogen limitation, accumulation of PHB and polysaccharide occured and that the dry-weight pattern was similar to that expected by theory (Herbert et al. 1956). This run was carried out in Fermentor 1 with a concentration of 190 ug nitrogen per ml. and the results shown in Tables 11 & 12. It was the first run carried out in the whole series and was the least satisfactory from the control point of view. However, as it was the first attempt by the author to grow an organism continuously, the experience gained ensured that there was no repetition of the serious problem of foaming that was encountered at low dilution rates during this run. As previously mentioned, a series-wound motor was used which added considerably to the problem of foaming. Antifoam was also added throughout, very high addition rates being necessary at low growth rates. The additional antifoam could, and indeed did, add to the dilution rate at D 0.085 $hr.^{-1}$. This meant that the dry weight was diluted by 5.5%, making 2.04 mg. per ml., as shown in brackets at D 0.085 $hr.^{-1}$ in Table 11.

Table 12

The Effect of Nitrogen Deficiency

Dilution Rate ($hr.^{-1}$)	0.085	0.2	0.2	0.35	0.5	0.65	0.8
% Polysaccharide Content (% of Dry Wt.)	16.4	13.0	14.9	13.5	12.4	11.0	8.1
% Glycogen Content (% of Dry Wt.)	5.5	6.8	6.0	4.75	4.75	1.8	1.6
Rate of Glycogen Production (ug/mg. dry wt./hr.)	4.7	13.6	12.0	16.7	23.8	11.7	12.8
% PHB Content (% of Dry Wt.)	10.0	9.7	10.0	14.7	16.4	11.9	10.6
Rate of PHB Production (ug/mg. dry wt./hr.)	8.5	19.4	19.9	51.5	81.7	77.4	84.7
% Phosphorus Content (% of Dry Wt.)	1.77	1.98	1.69	1.76	1.93	2.68	3.22
Glucose Yield Constant (mg. dry wt./mg. glucose)	0.15	0.27	0.33	0.42	0.37	0.39	0.44
Glucose Oxidation Rate (mg./mg. dry wt./hr.)	0.57	0.73	0.60	0.84	1.35	1.67	1.81
% Pyruvic Acid (% of Dry Wt.)	6.8	3.7	3.2	3.2	2.3	1.1	-
Rate of Pyruvate Production (ug/mg. dry wt./hr.)	8.0	7.4	6.4	11.2	11.5	8.8	-
% Oxoglutaric Acid (% of Dry Wt.)	17.6	7.4	8.6	7.7	6.7	6.3	-
Rate of Oxoglutarate Production (ug/mg. dry wt./hr.)	15.	14.8	17.2	26.9	33.5	40.9	-
% Acetic Acid (% of Dry Wt.)	6.6	8.4	14.0	9.9	20.1	30.0	-
Rate of Acetate Production (ug/mg. dry wt./hr.)	5.6	16.8	28.0	34.6	10	19.5	-

Dry Weight Yield

In the measurement of dry weight during this run the cells were washed with distilled water and not with 1% (W/V) NaCl as in later runs. This washing caused considerable lysis of the cells from the two highest dilution rates, and possibly resulted in a low dry weight per ml. Bearing this detail in mind, it is still apparent that the dry weight drops above D 0.5 $hr.^{-1}$. The yield figures are based on the total nitrogen in the ingoing medium as no suitable and safe device has yet been devised for direct sampling and filtering of this vessel. It is different for glucose limitation, larger amounts are available and time is not so critical in collecting the sample.

Mean Mass, Count, RNA & DNA Contents

The mean mass, reflecting the total count of organisms, showed little tendency to increase at low growth rates although there was apparently a minimum mass of 0.8 ug x 10^{-6}. From the amount of DNA per cell there appears to be only one nucleus at these low growth rates. The cells appear to contain at least two nuclei and possibly three at the highest rates of growth. The percentage content of RNA increased with increasing growth rate as expected, while the percentage of nitrogen reflects some

dilution of nitrogenous cell material at the lower growth rates.

Polysaccharide and Polyglucose

The total polysaccharide content was very high at low growth rates in comparison with other deficiencies. This was especially well shown in the polyglucose content of the cells. However, the results do not show a constant rate of polyglucose synthesis as found by Holme and Palmstierna (1957) for E. coli B; in fact there appears to be a point of maximum production at D 0.5 $hr.^{-1}$.

PHB

The PHB content showed a surprising pattern in both total content and rate of synthesis; a marked decline in content was noted below D 0.35 $hr.^{-1}$, the rate of production being constant between D 0.5 - 0.8 $hr.^{-1}$. The complex picture of possibilities presented by this pattern will be discussed later.

Phosphorus

The phosphorus content and volutin staining did not indicate any accumulation of phosphorus and polyphosphate.

Glucose Constants

The GYC increased, not unexpectedly, with

Table 13

The Effect of Nitrogen Limitation (2)

Dilution Rate ($hr.^{-1}$)	0.047	0.10	0.20	0.35	0.50	0.65	0.80	0.90
Doubling Time (hr.)	14.7	6.9	3.44	1.97	1.38	1.06	0.87	0.77
Dry Weight (mg./ml.)	0.82	0.80	0.78	0.78	0.78	0.775	0.75	0.715
Yield (mg. dry wt/mg. N.)	9.8	9.4	9.2	9.2	9.2	9.1	8.8	8.4
Rate of Cell Production (g./l./hr.)	0.04	0.08	0.16	0.27	0.39	0.51	0.60	0.64
Doubling Time when suspended in complete medium (hr.)	-	1.61	1.63	1.54	1.57	1.23	-	-
Polysaccharide Content (% of Dry Wt.)	-	13.5	12.3	11.2	-	9.8	10.2	10.0
Glycogen Content (% of Dry Wt.)	-	5.3	8.0	7.5	-	2.0	1.4	1.2
Rate of Glycogen Synthesis (ug/mg. dry wt./hr.)	-	5.3	16.0	27.2	-	13.0	11.2	10.8
PHB Content (% of Dry Wt.)	17.0	8.3	10.6	16.6	14.4	12.1	6.0	2.1
Rate of PHB Production (ug/mg. dry wt./hr.)	8.0	8.3	21.2	58.8	72.0	78.6	48	18.2
Rate of Depolymerisation in Washed Suspension (ug/mg. dry wt./hr.)	17.0	17.0	24.0	27.0	20.0	14.0	-	-
Glucose Yield Constant (mg. dry wt./mg. Glucose)	0.21	0.145	-	-	-	0.43	0.41	0.59
Glucose Oxidation Rate (mg. glucose/mg. dry wt./hr.)	0.22	0.69	-	-	-	1.51	1.93	1.53
Pyruvic Acid (% of Dry Wt.)	18.9	8.25	-	-	-	1.6	2.6	3.6
Rate of Production of Pyruvate (ug./mg. dry wt./hr.)	8.9	8.25	-	-	-	10.2	20.6	32.4
Oxoglutaric Acid (% of Dry Wt.)	45.8	18.4	-	-	-	7.6	6.6	3.3
Rate of Production of Oxoglutarate (ug./mg. dry wt./hr.)	21.5	18.4	-	-	-	45.0	50.3	30.0
Acetic Acid (% of Dry Wt.)	24.1	18.8	-	-	-	32.6	28.8	36.9
Rate of Production of Acetate (ug./mg. dry wt./hr.)	9.5	18.8	-	-	-	212	230	332

decreasing growth rate but the GOR indicated that the oxidation of glucose was related to the growth rate.

Overflow Products

Only those products identified in glucose deficiency were determined although formate was proved absent. Production of the three acids, pyruvate, oxoglutarate and acetate, occurred at all growth rates; the significance of the rates are discussed in relation to the results from the second nitrogen deficiency experiment.

Oxygen Demand

The oxygen demand for this culture was greater than that of glucose deficiency. This was not because the GOR was significantly different but because there was twice as much cell material per ml. The oxygen demand will therefore be slightly more than double at D 0.7 $hr.^{-1}$ in nitrogen deficiency than at D 0.7 $hr.^{-1}$ in glucose deficiency. This still leaves a large excess of oxygen supply which is not utilised.

Nitrogen Deficiency (Cont.)

The second nitrogen deficient run was carried out in Fermentor 2 at 85 ug N/ml., to attempt duplication of the first run and to determine the possible effect of a higher concentration of phosphate and the lack of antifoam.

As shown in Table 13, specific growth rates from 0.047hr.$^{-1}$ (one doubling in every 15 hr.) to 0.9 hr.$^{-1}$ (one doubling in every 46 min.) were obtained. Control of pH was fair being 7.3 $\pm$ 0.15 units, except at D 0.047 hr.$^{-1}$ where it was 7.08. Only a limited number of cellular components were followed during this run, primarily because the operator had insufficient time available to follow all the variables in two chemostats.

Dry Weight, Yield and Output

The dry weight per ml. shows remarkably little variation and reflects a consistant ability to produce medium of constant composition. This latter point has not been emphasised before, but it is imperative to find out exactly how much liquid is lost in evaporation during autoclaving and to use a constant time for this process. At low growth rates the Yield values show that nitrogen uptake was most efficient or that there was some dilution of nitrogen material. The output of cells increased almost uniformly with the dilution rate.

Polysaccharide and Polyglucose

Total polysaccharide was lower at low dilution rates than in the previous nitrogen deficiency experiment but the percentage of polyglucose was higher. This suggests

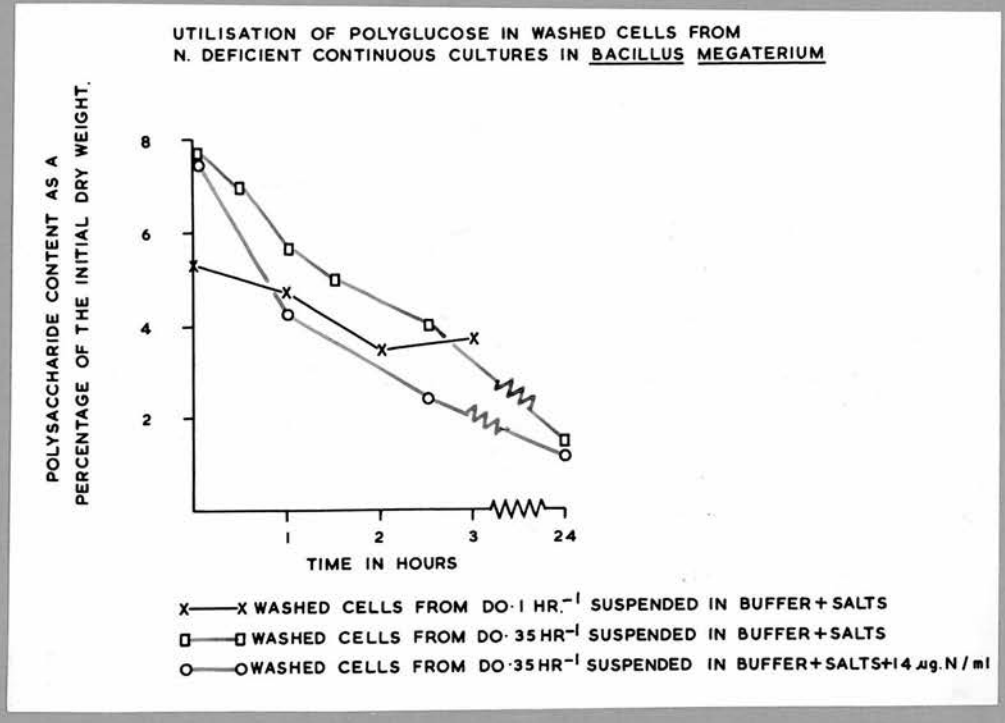

UTILISATION OF POLYGLUCOSE IN WASHED CELLS FROM
N. DEFICIENT CONTINUOUS CULTURES IN BACILLUS MEGATERIUM
POLYSACCHARIDE CONTENT AS A
PERCENTAGE OF THE INITIAL DRY WEIGHT.
8
6
4
2
1
2
3
24
TIME IN HOURS
X——X WASHED CELLS FROM DO·1 HR.⁻¹ SUSPENDED IN BUFFER + SALTS
□——□ WASHED CELLS FROM DO·35 HR⁻¹ SUSPENDED IN BUFFER + SALTS
○——○ WASHED CELLS FROM DO·35 HR⁻¹ SUSPENDED IN BUFFER + SALTS + 14 µg. N / ml

Fig. 29.

that phosphate concentration may affect not only the rate of synthesis of polyglucose but also the content of other polysaccharide components.

The rate of degradation of the polyglucose compound was followed in cells obtained from two dilution rates. Complete medium, without a carbon or nitrogen source, was used as suspending fluid. The effect of added nitrogen (14 ug N/ml.) was tested on cells from D 0.36 hr.$^{-1}$, the results of which are shown in Fig. 29. The presence of a nitrogen source apparently facilitated a more rapid breakdown of the polyglucose compound, perhaps by allowing balanced growth to occur. The faster grown cells can apparently utilise their polyglucose at a greater rate than those at D 0.1 hr.$^{-1}$.

PHB

The level of PHB was almost identical to that in the other nitrogen deficient run, but the two additional growth rates were of particular significance. At the lowest D value of 0.047 hr.$^{-1}$, the rate of PHB synthesis was similar to the previous rate at D 0.1 hr.$^{-1}$, resulting in a large increase in the total PHB content. The large increase in PHB perhaps accounts for the increased dry weight per ml. at D 0.047 hr.$^{-1}$. There was, at the fastest growth

rates, a definite decline in the rate of PHB synthesis. The rate of breakdown was measured as in glucose deficiency. However, the turbidities, after alkaline hypochlorite treatment at each dilution rate, were composed of two components, namely PHB granules and an insoluble material, whereas in glucose deficiency there were only PHB granules. Therefore, while the drop in turbidity was proportional to a drop in PHB content, the absolute turbidity value was not equivalent to the PHB content determined by the method of Law and Slepecky (1960b). The drop in turbidity in units per hr. was converted to μg per mg. dry weight per hr.

The rates of depolymerisation found by this method showed a maximum at D 0.35 $hr.^{-1}$ where synthesis was near maximal as well. However, at D 0.1 and D 0.047 $hr.^{-1}$, the rate of breakdown was faster than the rate of synthesis, meaning that either braakdown was not so fast, or does not occur during growth, or that the rate of synthesis was a composite figure of synthesis minus breakdown. Discussion of the choice of alternatives is presented later.

Glucose Constants

The GYC was similar to those results obtained in the previous run. The GOR, where measured, also sup-

ported the data from the previous N deficient run but it would appear that at approximately D 0.1 $hr.^{-1}$ and below, the GOR was nearly constant, hence the steady rate of PHB production.

Overflow Products

It is noticable from Table 13 that the rate of production of all the extracellular acids in nitrogen deficiency remained nearly constant at D 0.2 $hr.^{-1}$ and below. Pyruvate production increased suddenly where the rate of PHB synthesis started to fall. The amount of acetic acid formed at D 0.8 $hr.^{-1}$ was very similar to the amount formed during glucose deficiency at the corresponding rates of growth.

ATP ase Activity Experiments

In view of the fact that at D 0.2 $hr.^{-1}$ in nitrogen deficiency, the cells oxidised approximately twice as much glucose per unit of dry weight synthesised as in glucose deficiency, it might be expected that an excess of energy would be available. There are at least two possibilities to account for this. First, as a result of excess phosphorylation there would be an active demand for the regeneration of ADP and AMP from ATP, and secondly, uncoupling of oxidative phosphorylation might occur. Assuming that the

former was correct, one method for the cell to regenerate ADP is by ATPase activity. To test the ATPase hypothesis, cell-free extracts from three dilution rates (0.1, 0.2, and 0.65 hr.$^{-1}$) were prepared as in the methods section. The test involved measuring the inorganic phosphate liberated by this extract in the presence of ATP or ADP. No ATPase activity was detected; ADPase activities for the three D values measured were 0.21, 0.25, 0.16 μ mole of ADP phosphate liberated per mg. dry weight per hr. Considering that 3.5, 3.7 and 9.3 μ moles of glucose were oxidised per mg. dry weight per hr. and each μ mole may generate 36 μ mole of ATP this reaction was rather weak. The experiment indicates that if ATP were overproduced and ADP or AMP were in short supply, there was a low regeneration rate from ATPase or ADPase acitivity.

Measurement of Doubling Time in Batch Culture

Growth rates were recorded for most D values in batch culture experiments, as previously described. Cells from the slower growth rates do not reflect the rate at which they had been forced to grow in the chemostat, indicating that a slow strain had not been selected by the pressures in the vessel. However, there was a considerable difference between growth rates 0.1 - 0.5hr.$^{-1}$ and 0.65 hr.$^{-1}$ showing that some selection occurred.

Table 14

The Effect of Nitrogen Limitation + 0.1 M. Sodium Acetate.

Dilution Rate ($hr.^{-1}$)	0.2	0.35	0.5	0.65
Doubling Time (hr.)	3.44	1.97	1.38	1.06
Dry Weight (mg/ml.)	1.57	1.51	1.58	1.47
Yield (mg. dry wt/mg N)	8.7	8.4	8.8	8.2
Rate of Cell Production (g./l./hr.)	0.31	0.53	0.79	0.96
Population Density (Total count/ml. x 10^{-9})	1.25	1.25	0.96	0.55
Mean Cell Mass (ug. x 10^{-6})	1.26	1.21	1.65	2.68
% DNA Content (% of Dry Wt.)	1.96	2.10	1.77	1.74
DNA/cell (ug x 10^{-9})	24.6	25.5	29.4	46.6
% RNA Content (% of Dry Wt.)	5.0	7.6	12.8	17.4
RNA/cell (ug x 10^{-8})	6.3	9.2	21.1	47.0
% Nitrogen Content (% of Dry Wt)	11.4	11.8	11.7	11.4
% Polysaccharide Content (% of Dry Wt.)	6.8	6.8	5.3	6.4
% PHB Content (% of Dry Wt.)	1.05	0.88	1.8	1.4
% Phosphorus Content (% of Dry Wt.)	2.29	2.35	2.52	2.78

Nitrogen Limitation plus excess Acetate

This brief run was carried out after the first nitrogen limitation and was an attempt to define the role of excess acetate (0.1 M) in the presence of excess glucose on the cell content of polysaccharide and PHB. The run was carried out in Fermentor 1 with 180 ug nitrogen per ml. and the results are recorded in Table 14. This was the only other run where antifoam was added.

The dry weights were all very similar, the yield being very low and comparable with cells from the highest dilution rates of the other nitrogen deficiencies. Cell division was slightly inhibited and there was a resulting small build up of DNA per cell. The content of RNA was more depressed at D 0.2 and 0.35 $hr.^{-1}$ than in the corresponding values of nitrogen deficiency.

Acetate apparently suppressed the synthesis of PHB and polysaccharide and as a result the run was terminated at D 0.65 $hr.^{-1}$. Possibly the cell was forced to cut oxidative metabolism to a minimum. Therefore, the high rate of PHB synthesis in washed suspensions of cells suspended in excess glucose and acetate (Macrae and Wilkinson, 1958a) cannot be repeated in the chemostat where cells are growing. It is possible that in washed

cell suspensions some control mechanism was altered or damaged, allowing PHB synthesis to occur.

Table 15

The Effect of Potassium Limitation

Dilution Rate ($hr.^{-1}$)	0.1	0.2	0.35	0.5	0.65	0.8	0.83
Doubling Time (hr.)	6.9	3.44	1.97	1.38	1.06	0.8	0.83
Dry Weight (mg/ml.)	0.35	0.38	0.435	0.50	0.73	0.72	0.66
ug K/ml. (Ingoing Medium)	3.25	6.5	13.0	19.5	32.5	39	39
Dry Weight (Mg/ml.) (Based on 195 ug K/ml.)	2.10	1.14	0.65	0.50	0.44	0.36	0.33
pH of Culture	6.7	6.9	7.1	7.27	7.25	7.3	7.3
Yield(mg dry wt/mg K)	108	58	34	26	24	18	17
Rate of Production of Cell Material (g/l of culture volume/hr.)	0.21	0.23	0.23	0.25	0.39	0.29	0.25
Population Density Total Count/ml. x 10^{8}	2.60	3.16	3.78	3.62	2.35	1.90	-
Mean Cell Mass (ug x 10^{-6})	1.34	1.20	1.15	1.44	3.13	3.78	-
% DNA Content (% of Dry Wt.)	1.70	1.60	1.39	1.67	1.66	1.89	-
DNA/cell (ug x 10^{-9})	22.9	19.2	16.1	27.6	51.5	66	-
% RNA Content (% of Dry Wt.)	12.0	13.9	14.9	17.4	20.8	22.8	-
RNA/cell (ug x 10^{-8})	16.2	16.3	17.2	28.8	65	86.5	-
% Nitrogen Content (% of Dry Wt)	10.5	11.3	11.6	11.5	10.8	10.4	-

Potassium Deficiency

This run was carried out to investigate the effect of a deficiency of a metal cation on cell yield and cell physiology. The run was carried out in Fermentor 2 and the results are shown in Tables 15, 16. In this run it was found necessary to alter the concentration of potassium at each dilution rate in order to produce the minimum amount of organisms suitable for investigation. Low concentrations of cells were necessary in order to maintain as high a pH as possible, but even so the value dropped considerably at the lowest growth rates. The ingoing medium was at pH 7.8, the highest value permissible which did not cause precipitation of the manganese and magnesium, but even so, some precipitation did occur in the medium container and supply-line to the vessel.

Dry Weight, Yield, Cell Production Rate

The actual dry weights are shown with the corresponding concentration of K^+ per ml., and are also calculated on a basis of 19.5 ug K^+ per ml. The calculated dry weights from this fixed amount of potassium show a steep decline in both dry weight and yield, with increasing growth rate, although the rate of production of cells

Table 16

The Effect of Potassium Limitation (Cont.)

Dilution Rate ($hr.^{-1}$)	0.1	0.2	0.35	0.5	0.65	0.80	0.83
% Polysaccharide Content (% of Dry Wt.)	3.60	4.45	6.35	7.04	10.6	11.2	-
% Glycogen Content (% of Dry Wt.)	1.4	1.1	1.7	0.9	1.8	1.8	-
% PHB Content (% of Dry Wt.)	8.2	10.1	18.5	15.1	8.2	3.1	1.2
Rate of PHB Production (µg/mg. dry wt/hr.)	8.2	20.2	65.0	75.5	53.2	24.8	10.0
Glucose Yield Constant (mg. dry wt/hr. Glucose)	0.14	0.29	0.3	0.32	0.26	0.38	-
Glucose Oxidation Rate (mg. glucose/mg. dry wt/hr.)	0.72	0.69	1.17	1.56	2.50	2.1	-
% Pyruvic Acid (% of Dry Wt.)	5.35	2.9	1.4	2.2	1.5	5.8	-
Rate of Pyruvate Production (µg/mg. dry weight/hr.)	5.4	4.8	4.8	11	9.5	46.4	-
% Oxoglutaric Acid (% of Dry Wt.)	1.1	0.7	0.3	0.5	0.3	1.6	-
Rate of Oxoglutaric Production (µg/mg. dry weight/hr.)	1.1	1.4	1.1	2.5	2.0	12.6	-
% Acetic Acid (% of Dry Wt.)	271	183	98	87	51	46	-
Rate of Acetate Production (µg/mg. dry weight/hr.)	271	366	343	435	333	368	-

increased marginally. The picture is quite different to the increasing rates of cell production given by glucose or nitrogen deficiencies and in this respect the result was somewhat similar to that obtained by Postgate and Hunter (1962) for magnesium deficiency. The result could be interpreted as showing that the cell has a high Michaelis constant for the limiting substrate, potassium.

Mean Cell Mass and Nucleic Acids

The concentration of DNA per cell and the mean mass showed that little inhibition of cellular division occured at low growth rates. However, the cells did seem abnormally large at the fastest rates of growth, and the content of DNA and RNA per cell were the highest recorded at these growth rates.

Polysaccharide and Polyglucose

Potassium deficiency is known to be specifically inhibitary to polysaccharide synthesis in both Diplococcus pneumoniae (Bernheimer, 1953) and A. aerogenes (Wilkinson, Duguid and Edmunds, 1954). In the present work it can also be seen that, at low growth rates, there was a reduction of the total content of polysaccharide. The formation of polyglucose was almost totally suppressed at all growth rates. In order to determine whether any soluble

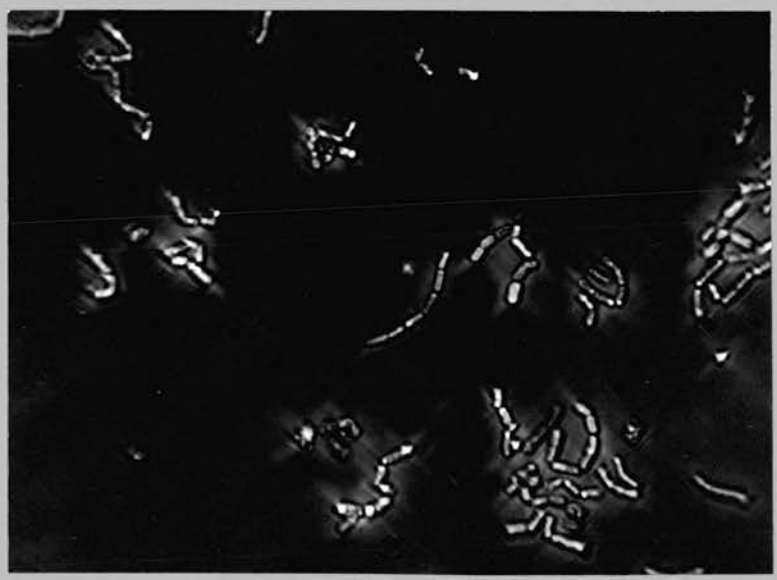


Fig. 30: Photomicrograph of Bacillus megaterium showing chain formation. Potassium limitation, specific growth rate 0.2 hr.$^{-1}$, water mount, medium dark phase contrast x 100

polysaccharides were produced into the medium, the spent culture was collected from D 0.2 $hr.^{-1}$. It was dialysed against tap water for 48 hr., concentrated against polyethylene glycol and examined for polysaccharide content, but none was present.

As previously mentioned smooth colonies were detected at growth rates of D 0.35 $hr.^{-1}$ and below. During these same growth rates noticable chain-formation occurred, as shown in the photomicrograph in Fig. 30. Chain formation is certainly related to cell wall structure and possibly to polysaccharide synthesis but the connection with potassium deficiency is not obvious.

PHB

The content of PHB and its rate of synthesis were most similar to those in nitrogen deficiency, the maximal content and rate of synthesis occurred at much the same rates of growth. The rate of production of PHB fell rapidly at faster growth rates.

GYC and GOR and Overflow Products

Both the GYC and GOR were very similar to those found for nitrogen deficiency. The fact that the GOR was similar to nitrogen deficiency and that vast amounts of acetate accumulated suggests that the pathway which oxidises this acetate in nitrogen deficiency is at some considerable

disadvantage in potassium deficiency. Oxoglutarate production reached the lowest levels yet noted, suggesting that the TCA cycle is involved in oxidation of acetate and that the nonentry of acetate resulted in the low production of oxoglutarate. Pyruvate production was not noticably affected by potassium limitation when compared with the values for the other runs.

Table 17

The Effect of Sulphur Limitation

Dilution Rate (D) ($hr.^{-1}$)	0.11	0.20	0.30	0.39	0.40	0.50	0.50	0.60	0.66	0.80
Doubling Time (hr.)	6.3	3.44	2.31	1.77	1.73	1.38	1.38	1.15	1.04	0.86
Dry Weight (mg/ml.)	1.31	1.70	1.68	1.37	1.29	1.01	1.08	0.83	0.49	0.07 (0.505)
Yield (mg. dry wt/mg of Element)	383	496	490	400	376	295	317	242	144	20.5
Rate of Cell Production (g/l. of culture volume/hr.)	0.14	0.34	0.51	0.53	0.52	0.51	0.54	0.50	0.32	0.06
Population Density Total Count/ml. x 10^8	2.82	3.08	8.0	-	7.15	6.86	-	5.1	2.54	1.60
Mean Cell Mass (ug x 10^{-6})	4.4	5.51	2.1	-	1.81	1.47	-	1.63	1.93	3.20
% DNA Content (% of Dry Wt.)	1.15	1.20	1.38	-	1.44	1.66	-	1.41	1.83	1.89
DNA/cell (ug x 10^{-9})	58.5	66.6	29.0	-	25.9	24.5	-	23.0	35.4	60.0
% RNA Content (% of Dry Wt.)	7.6	7.9	9.0	-	11.2	13.4	-	17.0	19.5	22.1
RNA/cell (ug x 10^{-8})	35.4	43.6	18.9	-	21.5	19.9	-	27.7	37.6	69.8
% Nitrogen Content (% of Dry Wt.)	11.05	10.1	11.6	-	11.2	11.2	-	11.4	11.9	11.7

Sulphur Limitation

This run was carried out to investigate the effect of sulphur deficiency on cell yield and physiology. The run was carried out in Fermentor 1 and the results shown in Tables 17 and 18.

Dry Weight, Yield and Cell Production Rate

The yield figures obtained were very high at low growth rates, almost twice as high as those of Postgate and Hunter (1962) for sulphur limitation with A. aerogenes. Only when the high rate of cell production is examined do the results of dry weight appear to have some meaning. The rate of production increased rapidly from D 0.1 - 0.3 $hr.^{-1}$ in a manner similar to that in nitrogen deficiency, perhaps indicating that all the sulphur source was utilised, i.e. nearly all the sulphur found inside the cells. The second stage, from D 0.3 - 0.6 $hr.^{-1}$, was most similar to potassium deficiency. If the dry weight per ml. is plotted against the doubling time for both potassium and sulphur deficiencies, the results given in Fig. 31 show that the potassium deficiency from D 0.1 - 0.8 $hr.^{-1}$ and the sulphur deficiency between D 0.3 - 0.6 $hr.^{-1}$ both appear to stem from the origin. A possible similarity in

TABLE 18

THE EFFECT OF SULPHUR LIMITATION (CONTD.)

Dilution Rate($hr.^{-1}$)	0.11	0.2	0.3	0.39	0.40	0.50	0.50	0.60	0.66	0.80
Polysaccharide Content (% of Dry Wt.)	6.0	7.4	9.8	-	10.9	12.5	-	12.5	10.9	12.5
Glycogen Content (% of Dry Wt.)	1.2	2.0	3.0	-	1.2	1.0	-	1.3	1.2	0.7
Rate of Glycogen Production (µg/mg. dry wt/hr.)	1.32	4.0	9.0	-	4.8	5.0	-	7.8	7.9	5.6
PHB Content (% of Dry Wt.)	14.3	15.1	14.5	11.0	13.1	8.5	7.0	6.7	6.8	3.5
Rate of PHB Production (µg/mg. dry wt/hr.)	15.8	30.2	43.5	43.0	52.5	42.3	35.0	40.0	45.0	28.0
Phosphorus Content (% of Dry Wt.)	1.61	1.63	1.78	-	2.17	2.31	-	2.77	2.71	3.66
Glucose Yield Constant (mg. dry wt/mg glucose used)	0.35	0.28	0.39	-	0.29	0.28	0.30	0.32	0.27	0.29
Glucose Oxidation Rate (mg. Glucose/mg. dry wt/hr.)	0.31	0.70	0.77	-	1.4	1.7	1.7	1.8	2.4	2.8
Pyruvic Acid (% of Dry Wt.)	3.2	2.2	1.7	-	2.0	2.3	-	2.8	3.1	5.5
Rate of Pyruvate Production (µg/mg. dry wt/hr.)	3.5	4.4	5.1	-	11.6	11.5	-	16.8	20.4	44.0
2-oxoglutarate (% of Dry Wt.)	3.3	2.9	1.3	-	1.7	2.5	-	4.4	5.8	6.7
Rate of Oxoglutarate Production (µg/mg. dry wt/hr.)	3.3	5.8	3.9	-	6.8	12.5	-	26.4	38.4	54.0
Acetic Acid (% Dry Wt.)	62.3	44.9	79.5	-	77.4	71.4	-	76.0	66.0	60.0
Rate of Acetic Acid Production (ug/mg. dry wt/hr.)	68.5	89.8	238	-	310	357	-	456	436	483

function, mode of entry, or participation in a growth-rate-limiting reaction are perhaps indicated.

A straight line drawn through D 0.6 - 0.8hr.$^{-1}$ appears to pass near or through the maximum growth rate. At least two possible reasons for this behaviour are evident:- (1) a permease system limited entry of SO_4^{2-} into the cell or (2) some step in SO_4^{2-} reduction became rate limiting.

Mean Cell Mass

During the lowest three growth rates the mean cell mass was very high. The cells were ovoid rather than rod shaped, just as those in glucose deficiency plus acetate at these three growth rates, but from D 0.4 - 0.8 hr.$^{-1}$ they followed closely the regular pattern.

Nucleic Acids

At the lowest growth rates there was apparently no shortage of DNA per cell as the cells contained approximately four nuclei. Cell size and the nuclear content dropped at D 0.35 - 0.5 hr.$^{-1}$, but above D 0.5 hr.$^{-1}$ the nuclear content per cell rose again, just as in other deficiencies. The RNA content per cell was much higher than in the glucose deficiency plus acetate at low D values. As in potassium

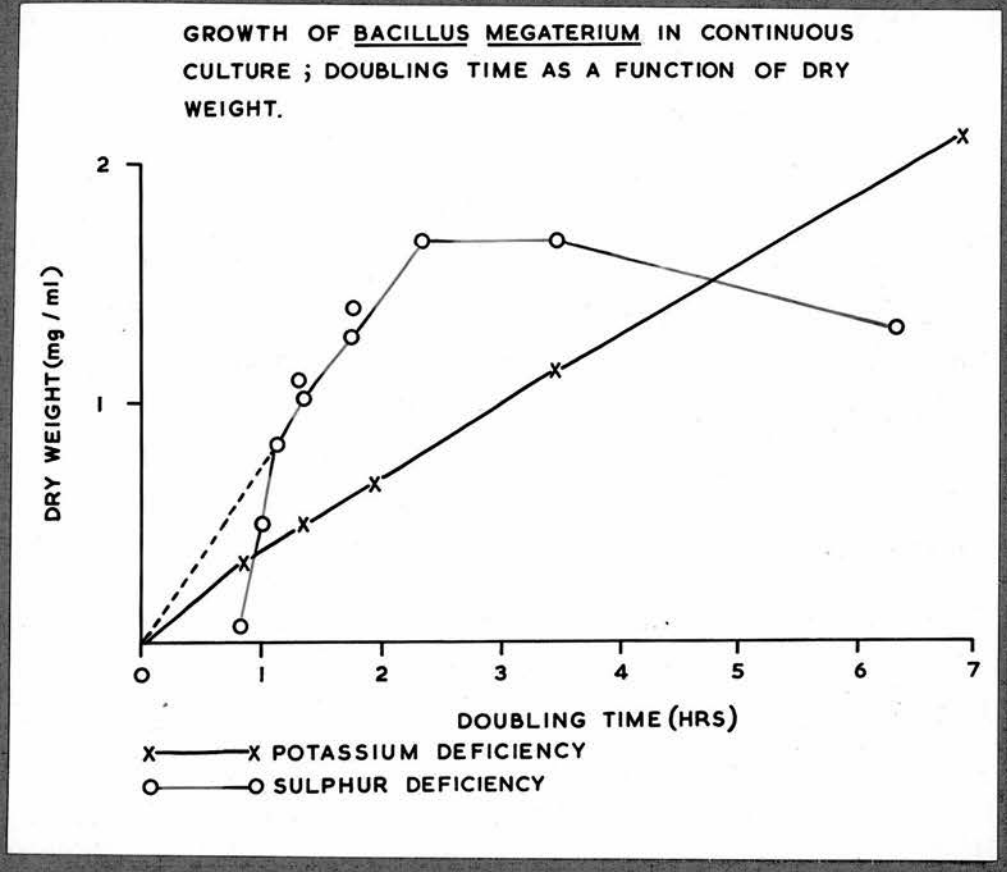

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE ; DOUBLING TIME AS A FUNCTION OF DRY WEIGHT.
DRY WEIGHT (mg / ml)
2
1
0
1
2
3
4
5
6
7
DOUBLING TIME (HRS)
X——X POTASSIUM DEFICIENCY
O——O SULPHUR DEFICIENCY

Fig. 31.

and nitrogen deficiencies there was approximately ten times more RNA per cell than DNA.

Polysaccharide and Polyglucose

The percentage of polysaccharide was low at D 0.1 and D 0.2 $hr.^{-1}$, but it was twice the amount found in glucose deficiency plus acetate. However, from D 0.4$hr.^{-1}$ the polysaccharide content ranged from 10 - 12.5% of the dry weight which was quite high although the values were not reflected in the polyglucose analysis. The polyglucose results were in agreement with sulphate-limited batch culture experiments of Holme and Palmstierna (1956b) on *E. coli* B, who concluded that sulphur starvation affected some basic mechanism in cell metabolism.

PHB

Staining the cells with Sudan Black at D 0.1 and D 0.2 $hr.^{-1}$ when the cells were very large did not give a similar general staining of the cytoplasm as in glucose deficiency plus acetate. Large discrete granules could be observed. The chemical analysis showed that the percentage of PHB was large at the low growth rates, higher than in nitrogen or potassium deficiency. The maximum rate of synthesis of PHB was however, slightly lower than in glucose

deficiency, and the rate of synthesis fell at the fastest rates of growth.

GYC, GOR and Overflow Products

The GYC was very high in this deficiency at low growth rates and this is reflected by a low GOR. At low D values a chronic shortage of sulphur could easily be expected to depress the rate of glucose metabolism by shortage of necessary sulphydryl groups. Acetic acid was produced in fair quantity at low growth rates, its overall production probably being limited by the low GOR. Production of the two oxoacids was not high until D 0.6 - 0.8 $hr.^{-1}$ where acetic acid production was at the highest values yet recorded.

Phosphorus and Volutin Staining

There were no significant signs of polyphosphate accumulation in the figures for the percentage of phosphorus or in the volutin staining. However, in view of the fact that both Smith, Wilkinson and Duguid (1954) and Harold (1960) found that cells starved of sulphur accumulated polyphosphate, albeit in the stationary phase in batch culture, a fractionation procedure for phosphorus compounds was carried out according to the less tedious method of Harold (1960). The results of the analysis of two dilution rates are shown in Table 19.

Table 19

Fractionation of Phosphorus-containing compound in Sulphur deficient cells.

Results are mg. phosphorus/100mg. dry weight.

Fraction	Dilution Rate	
	0.1	0.2
Total Phosphorus/100mg. dry weight	1.61	1.63
Cold Perchloric Acid Extract		
Total Phosphorus*	0.520	0.488
Orthophosphate	0.462	0.427
Acid-labile phosphate (Polyphosphate)	0.012	0.007
∴ Nucleotide Phosphate	0.047	0.054
Ether Soluble Extract		
Total Phosphorus*	0.072	0.062
Hot Perchloric Acid Extract		
Total Phosphorus*	0.841	1.060
Acid-labile phosphate (Polyphosphate)	0.342	0.091
∴ Nucleic Acid Phosphate	0.499	0.969
Residue		
Total Phosphorus*	0.092	0.071
Recovered*	1.525	1.681

The fractionation was repeated on cells from D 0.1, 0.2 and 0.5 hr.$^{-1}$, but only the acid labile phosphate in the hot acid extract was measured. The equivalent amounts were 0.293, 0.144 and 0.150 mg. phosphorus per 100 mg. of cells respectively. An unsatisfactory aspect of this method is the uncertainty about the nature of the material termed polyphosphate. However, if taken at their face value, the results show that an increased amount of polyphosphate accumulated at D 0.1 hr.$^{-1}$. This can easily ebe explained by the findings of Harold and Sylvan (1963) that low levels of oxidised glutathione allow formation of polyphosphate. Presumablyk at faster growth rates, higher levels of glutathione were present and this prevented formation of polyphosphate.

From the small amounts of polyphosphate found in the present series of results it would be difficult to justify the view that in B. megaterium polyphosphate is a phosphorus storage compound (Harold and Sylvan, 1963), or even an energy store. Harold (1963) has shown that when nucleic acid synthesis is occuring, polyphosphate levels are suppressed, therefore, only investigation of very low specific growth rates, much below 0.1 hr.$^{-1}$, where nucleic acid synthesis rates are low, would perhaps give high levels

of polyphosphate. An interesting observation is that in sulphur deficiency where there sould be a low level of intermediates such as oxidised glutathione, the substance controlling polyphosphate levels (Harold and Sylvan, 1963), there is an increase in content of RNA per cell at D 0.1 - 0.2 $hr.^{-1}$. This suggests that the level of oxidised glutathione may play a dual role controlling to some extent RNA synthesis at lower growth rates.

VII

DISCUSSION

DISCUSSION

The information gained in this study may be considered in three aspects.

1. The genetic stability of the organism used, i.e. the ability to maintain the observed characteristics of the strain in the chemostat.
2. The relation between the limiting nutrient and the dry weight, yield and output of material.
3. The effect of the growth rate and the limiting substrate on:-
   a. cell size and cell mass.
   b. nucleic acid content.
   c. possible storage compounds.
   d. oxidation of carbon substrate and production of extracellular products.

1. Selection

The problem of genotypic selection is very real in any continuous culture system. The initial inoculum contains a multitude of different genotypes and as time passes and successive generations multiply, further mutations will occur. However, most mutations will be of a lethal nature, especially those occurring in a simple salts medium. The repeated displacement of an existing population by fitter

types was regarded by Atwood, Schneider and Ryan (1951) as a mechanism of homeostasis, which means that if a mutant grows faster than the parent it will replace it at an exponential rate. If the mutant grows slower than the parent and if the mutation is greater than the rate of back mutation, a low constant level of the mutant will be observed. This indeed happened in our experiments as is evidenced by the continual production of a very rough colonial form which was always present in smaller amounts than the parent. The appearance of sporing strains in sulphur deficiency and the production of smooth colonies in potassium deficiency can also be explained in this manner, but the disappearance of the smooth strains at growth rates above D 0.35 $hr.^{-1}$ must only mean that conditions were unfavourable for their survival.

The aforementioned changes to very rough and smooth colonies and to sporing forms are different expressions of genetic character which are possibly of episomal nature. The high occurrence of colonial variants is a bacterial character which Jacob, Schaeffer and Wollman (1960) suggested is episomally controlled because these changes do not correspond to essential cellular functions, but rather to functions which can be superimposed on the normal metabolism of the cell. These

authors suggest that, under various conditions, such as changes in cationic balance of various types of starvation, the episome becomes detached and therefore activated or unrepressed. The various functions then controlled by the limiting nutrient express themselves with concomitant drastic changes in the morphology, composition, and metabolism of the cell.

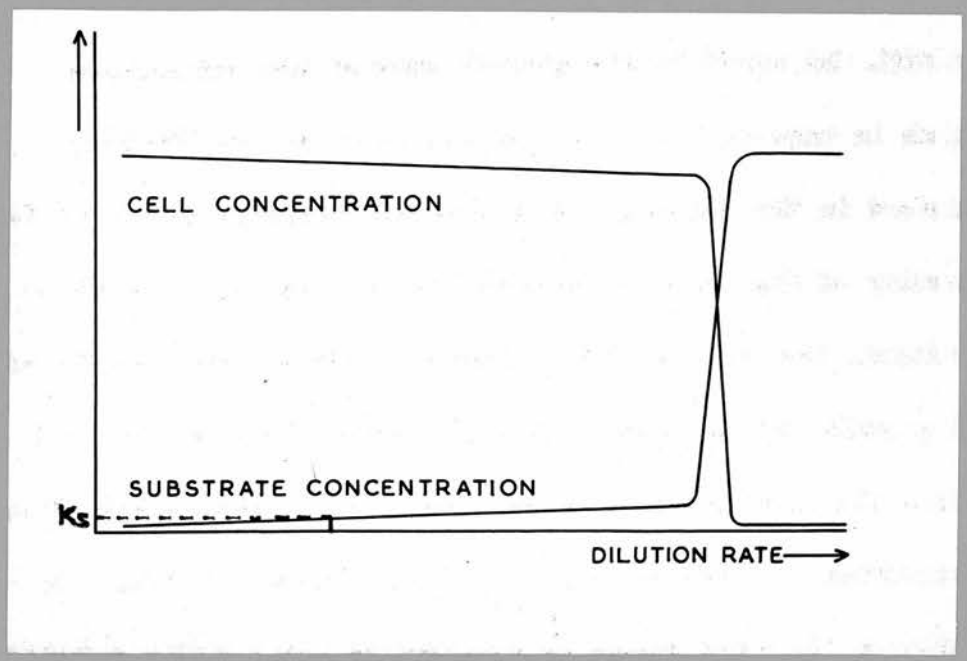
In the glucose-deficiency run, positive evidence of selection is shown because the doubling times of cells from different dilution rates grown in batch culture are different. These doubling times are not as long as the corresponding doubling time in the vessel, but there is sufficient difference between cells from consecutive dilution rates to show that the lower the D value, the longer the doubling time in batch culture. If the organisms from each dilution rate were identical genetically, then according to the up-shift experiments of Kjeldgaard et al. (1958) the cells might be expected to take up their new division rate several minutes after transfer to the complete medium which, because all nutrients are in excess, should allow a doubling time of near 60 - 70 min. Adaptation from a slow rate should be a quick process and should have occurred in the 2 - 3 hr. period before turbidity

measurements were carried out. Some adaptation must have occurred because the doubling times recorded in batch culture were much faster than the doubling time from their respective dilution rate in the culture vessel. It is perhaps unfortunate that the batch culture experiments on doubling times were not continued for much longer periods to ascertain if the low growth rate of the strain was stable to subculture. The results of the nitrogen-deficiency confirm that adaptation from low D values can be quick and the growth rate adopted be near the maximal for the organism although some selection of slower growing strains must have occurred below D 0.65 $hr.^{-1}$. Novick and Szilard (1950b) claim to have found faster growing strains selected in more complex media. Therefore, it would appear that the results of the glucose-deficiency show that selection of genotypically different organisms occurred at each dilution rate. The fastest growth rates achieved in the chemostat are something of a mystery. Only after growth at low D values will the organism grow faster than D 0.65 $hr.^{-1}$. The change in the ability to grow at faster rates after growth at slow rates is independent of the limiting nutrient. This could easily be explained by a genetical change at the low growth rate. However, we have been unable to grow the organism in batch culture at

specific growth rates above 0.65 hr.$^{-1}$, suggesting that adaptation occurs above this point only in the culture vessel. One feature of the batch culture experiments is that they are carried out in a 'shake-flask' in which oxygen transfer rates must be very low in comparison to those occurring in the vessel; even so that culture density was at least twenty times more dilute. Until the experiments on a fast growing strain can be repeated in a vessel with a demonstratably high oxygen transfer rate, too much should not be read into their meaning. The inability of the parent strain to grow above a specific growth rate of 0.65 hr.$^{-1}$ must be considered as additional evidence that genetic selection is occurring.

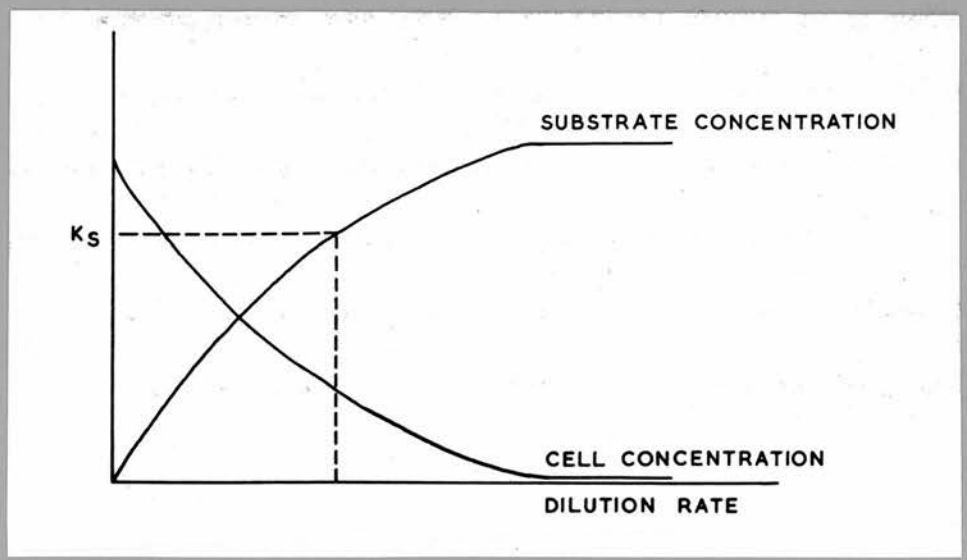
From the results of the chemical analyses it would be quite misleading to suppose that selection of mutants occurred. At the present stage it is quite impossible to decide what difference, if any, would exist between two organisms which are favoured by different dilution rates, but are grown at similar rates of growth. Unless the mutation gave an all or none change, as that described by Pirt, Thackeray and Harris-Smith (1961) for antigen production in Pasteurella pestis, it is doubtful whether differencies in chemical analyses from material collected from similar growth rates but at different times could

Fig. 32: Theorectical changes in cell concentration and substrate concentration with dilution rate assuming that the curve of substrate concentration is the mirror image of the curve of cell concentration:-



a.

where the inflowing substrate concentration is much larger than K_s.



b.

where K_s is greater than half the inflowing substrate concentration.

decide if a population had been supplanted by another.

2. The relation between the limiting nutrient and the dry weight, yield and output of cell material.

The theory of operation of the chemostat demands that the dilution rate, when 'steady state' conditions prevail, be equal to the growth rate of the organisms which in turn defines the concentration of the limiting nutrient in the vessel. In a simple medium, provided the quantity of the limiting nutrient in the ingoing medium is constant, the nature of the limiting nutrient will define at all growth rates, except perhaps those near the washout point, the growth pattern, the yield and the output of cells. Theoretical curves of dry weight are shown in Fig. 32a where K_s is very small in relation to the ingoing substrate concentration and in Fig. 32b where K_s is greater than half the ingoing substrate concentration. In both these figures it is worth emphasising that the curve of the limiting substrate concentration is represented as being the mirror image of the growth pattern (dry weight per ml.).

In these theoretical diagrams the mass of organisms per ml. has a maximum value when the dilution rate is zero, the substrate concentration then being zero.

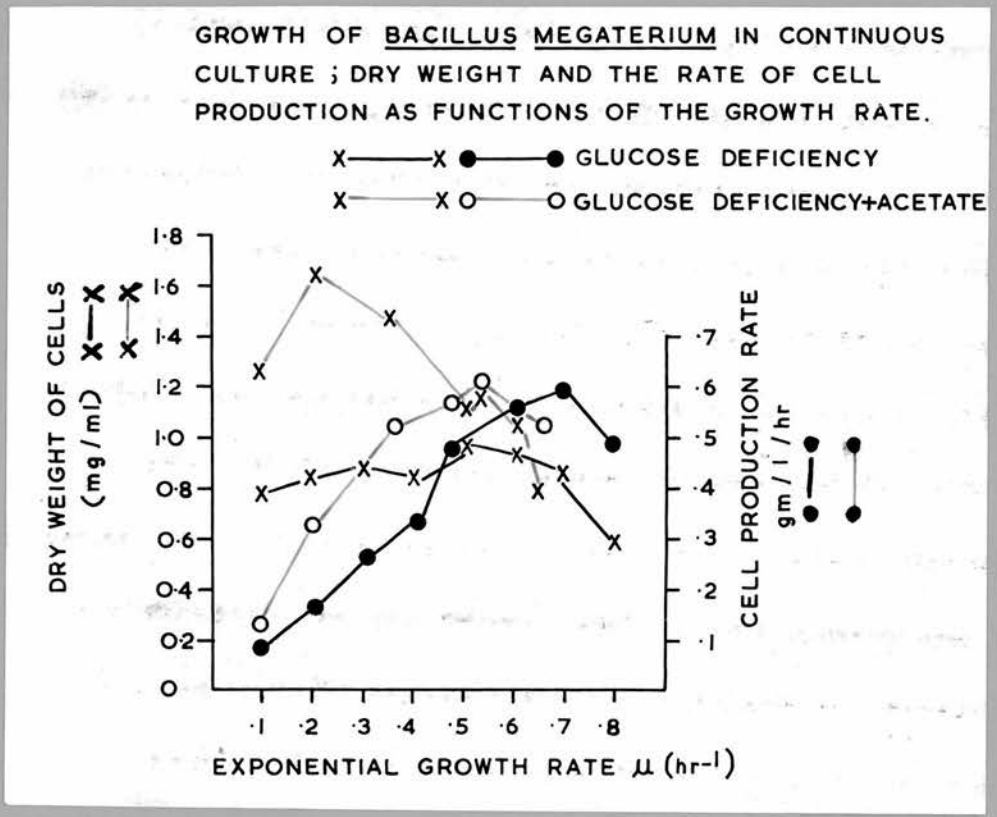

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE ; DRY WEIGHT AND THE RATE OF CELL PRODUCTION AS FUNCTIONS OF THE GROWTH RATE.
GLUCOSE DEFICIENCY
GLUCOSE DEFICIENCY+ACETATE
DRY WEIGHT OF CELLS (mg/ml)
CELL PRODUCTION RATE gm/l/hr
EXPONENTIAL GROWTH RATE μ (hr-1)
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0
·7
·6
·5
·4
·3
·2
·1
·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8

Fig. 33.

As an example of carbon limitation the dry weight and output curves for glucose deficiency are shown in Fig. 33. The curves vary from the theoretical form, especially at low growth rates. Necessary cellular activities, such as turnover of nucleic acids and protein molecules, and osmotic regulation and maintenance of intracellular pH, are supplied by energy from two possible sources; in the non-growing cells from the endogenous metabolism and in growing cells, partly, if not wholly, from the catabolism of exogenous substrate. It would seem reasonable to assume that the results show that a portion of the external energy source provides this sustaining energy with the consequent drop in yield observed at low growth rates. The results are not sufficiently numerous to decide if the maintenance requirement is constant per unit of dry weight per hr. Only more results, especially in the region below D 0.1 $hr.^{-1}$ where yields will be much less, will decide if the maintenance requirement is constant. The picture shown in lactate and succinate limitations is similar to that of glucose deficiency but glycerol limitation is different, the implications of which have been discussed previously.

Acetate uptake during glucose limitation (Fig. 33) apparently depends on intermediates and energy from

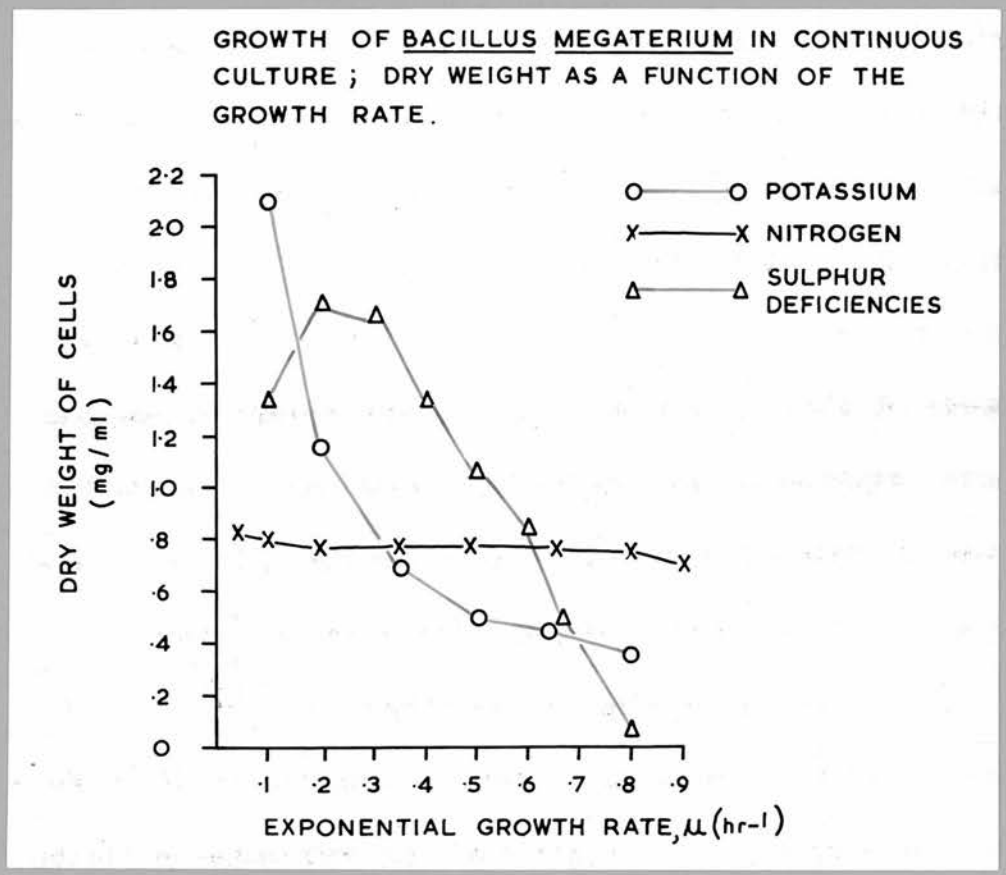

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE ; DRY WEIGHT AS A FUNCTION OF THE GROWTH RATE.
DRY WEIGHT OF CELLS (mg/ml)
2·2
2·0
1·8
1·6
1·4
1·2
1·0
·8
·6
·4
·2
0
·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9
EXPONENTIAL GROWTH RATE, μ (hr⁻¹)
POTASSIUM
NITROGEN
SULPHUR DEFICIENCIES

Fig. 34.

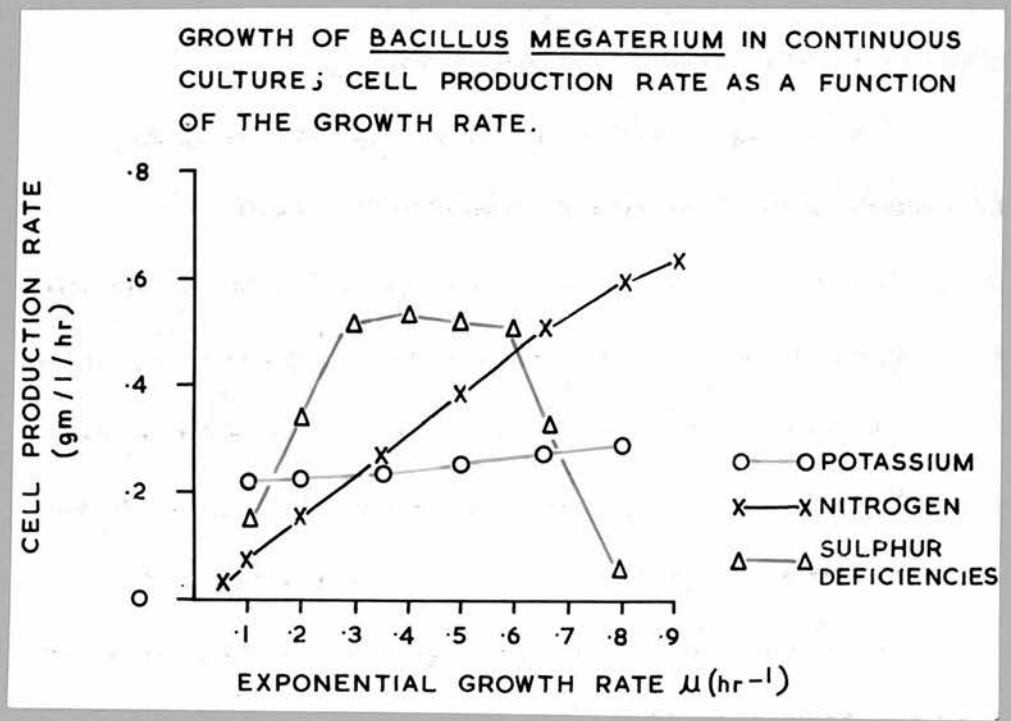

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE; CELL PRODUCTION RATE AS A FUNCTION OF THE GROWTH RATE.
CELL PRODUCTION RATE (gm/l/hr)
·8
·6
·4
·2
0
·1 ·2 ·3 ·4 ·5 ·6 ·7 ·8 ·9
EXPONENTIAL GROWTH RATE μ (hr⁻¹)
POTASSIUM
NITROGEN
SULPHUR DEFICIENCIES

Fig. 35.

glucose metabolism since at low D values the rate of cell production approaches that of glucose deficiency. The most probable reason for this is that acetate uptake is dependent on the catabolic metabolism of glucose for intermediates and energy, these becoming progressively lower at slow growth rates due to the energy of maintenance requirement proportionally increasing. The maximum rate of acetate incorporation occurs at D 0.34 $hr.^{-1}$ and drops at faster growth rates. The drop is probably caused by the increasing concentrations of glucose or a derived intermediate, e.g. acetate, repressing the acetate permease system. In support of this argument it should be remembered that in glucose deficiency internal acetate concentrations result in a measureable amount of acetate appearing in the medium at D 0.5 $hr.^{-1}$.

With nitrogen limitation (Fig. 34) the variation in cell density with flow rate is practically identical to Fig. 32a where K_s values are very low. This means that the nitrogenyields, that is the fraction of the ingoing nitrogen in the medium utilised to give cell material, are very high. The plot of cell production (Fig. 35) against dilution rate is a straight line passing through the origin.

The pattern of dry weight against the flow rate for potassium deficiency (Fig. 34) resembles Fig. 32b, where

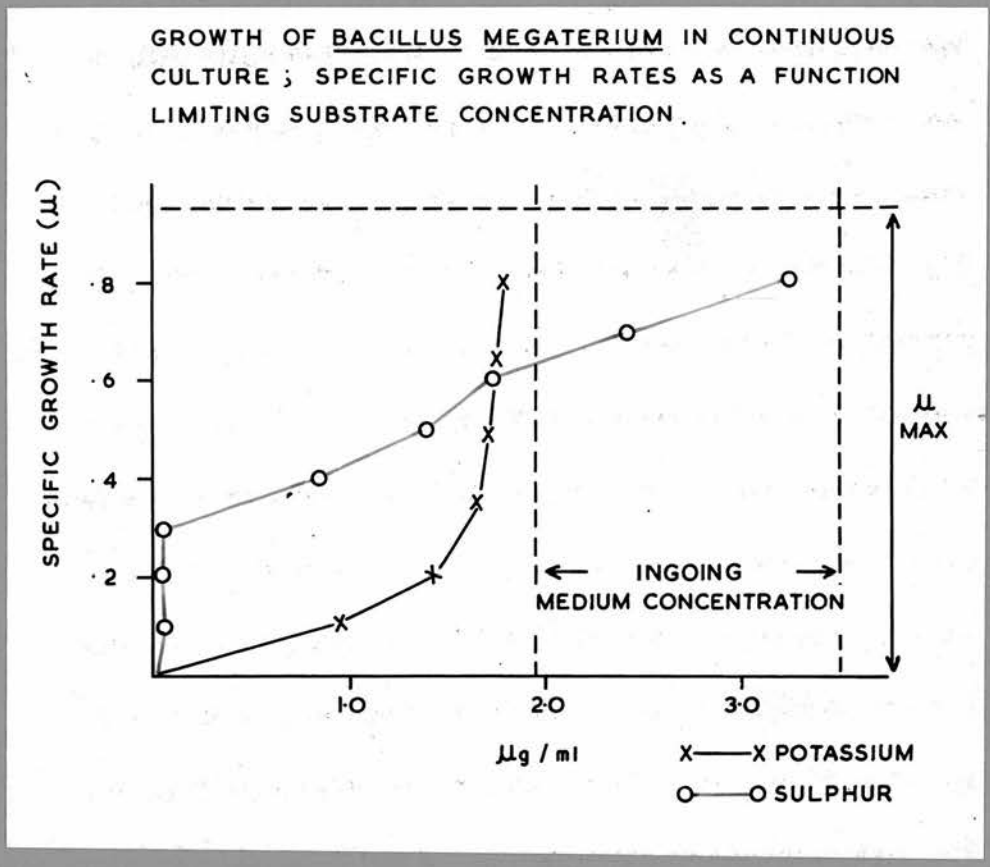

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE ; SPECIFIC GROWTH RATES AS A FUNCTION LIMITING SUBSTRATE CONCENTRATION.
SPECIFIC GROWTH RATE (μ)
·8
·6
·4
·2
μ MAX
INGOING MEDIUM CONCENTRATION
1·0
2·0
3·0
μg / ml
X——X POTASSIUM
O——O SULPHUR

Fig. 36.

K_s in assumed to be very large. Assuming that the content of potassium in the cell does not alter at each growth rate (this is perhaps questionable) and that for a figure for potassium content we use half the concentration of K^+ which gives 2.1 mg. dry weight per ml. at D 0.1 $hr.^{-1}$, we can calculate the content of potassium ions per ml. in the culture in the vessel. The specific growth rate is shown as a function of this potassium concentration in Fig. 36, or in other words, as a rate of reaction against substrate concentration. However, the plot obtained is not similar to a Michaelis graph as nitrogen and glucose limitation would be. Reversing Fig. 36, so that the potassium ion concentration per ml. is a function of the dilution rate, we would obtain a similar plot to substrate concentration in Fig. 32b and a mirror image of the curve of dry weight per ml. for K^+ limitation shown in Fig. 34. The cell production rate in potassium limitation (Fig. 35) shows a slight rise with increasing D value but is in marked contrast to the sharp rise shown by carbon and nitrogen limitation at equivalent rates of growth.

A possible reason for the peculiar pattern of dry weight and output of cells is that little or no potassium will be bound by covalent linkages as atoms of carbon, nitrogen or sulphur. These latter entities will probably become

covalently linked during entry into the cell whereas potassium will always be found in the free ionic state. The cells will still concentrate potassium but the chances of achieving efficient concentration, and hence nearly 100% removal from the culture medium as in nitrogen or carbon deficiency, will be low because the increasing concentration in the cell is likely to inhibit the reaction. This means that to effect a faster growth rate, a substantial increase in the external concentration of potassium ions is necessary, but since the external concentration may be a significant portion of the total inflowing potassium ions there is a resultant drop in the dry weight per ml. Further speculation would seem irrelevant until the potassium concentration is measured in the cells or in the effluent to confirm that the curves of dry weight per ml. and the concentration of potassium per ml. are in fact mirror images.

Sulphur deficiency shows a different curve of dry weight with flow rate (Fig. 34). Cell output is shown in Fig. 35. Assuming the sulphur content of cells grown at all growth rates to be similar and that virtually all the sulphur is found in the cells between D 0.1 - 0.3 $hr.^{-1}$, the growth rate is shown as a function of the sulphur concentration in the vessel (Fig. 36). From this last mentioned

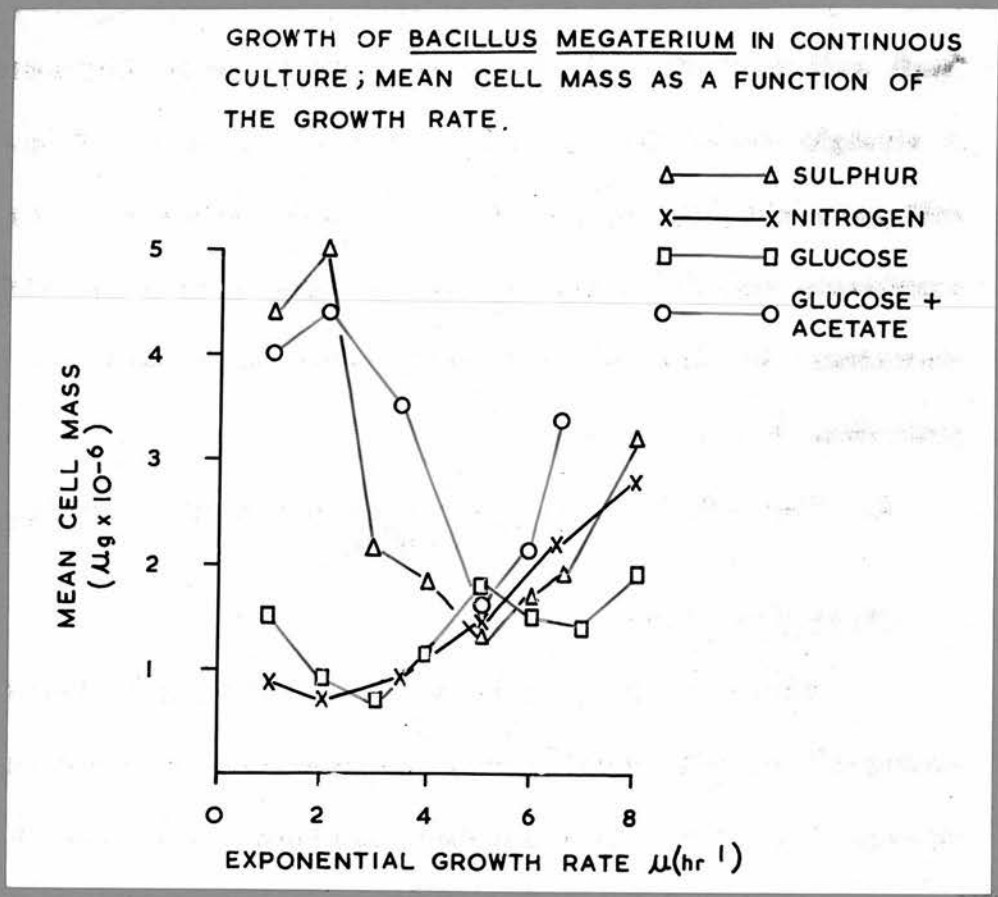

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE; MEAN CELL MASS AS A FUNCTION OF THE GROWTH RATE.
SULPHUR
NITROGEN
GLUCOSE
GLUCOSE + ACETATE
MEAN CELL MASS (μg x 10⁻⁶)
1
2
3
4
5
0
2
4
6
8
EXPONENTIAL GROWTH RATE μ(hr⁻¹)

Fig. 37.

figure it will be seen that the curve of sulphur content per ml. resembles that depicted in Fig. 32a, except that the point of inflexion occurs at a lower growth rate than in nitrogen or glucose limitation. The output of cells (Fig. 35) illustrates that probably at least three mechanisms limit cell production during growth on sulphate limitation. A straight drawn through points D 0.1, 0.2 and 0.3 $hr.^{-1}$ will pass near the origin as in nitrogen deficiency. As previously mentioned in the results section under sulphur limitation, at least two different mechanisms limit the yield from D 0.3 - 0.8 $hr.^{-1}$.

3. The effect of the Growth Rate and the Limiting Nutrient

a. Mean Cell Mass

The cell mass appears to exhibit certain features during all growth deficiencies, comparisons being shown in Fig. 37. There is a marked tendency for a drop in cell size from D 0.8 - 0.5 $hr.^{-1}$ in all deficiencies. Below D 0.5 $hr.^{-1}$ it is a feature of the limitation whether cell size increases as in sulphur or glucose limitation plus acetate, or continues to drop below D 0.5 $hr.^{-1}$ reaching a minimum at D 0.2 - 0.3 $hr.^{-1}$ as in glucose, nitrogen, or potassium limitation. At D 0.2 $hr.^{-1}$ in glucose and potassium limitations, size increases again, possibly indi-

cating some inhibition of division. The increasing size below D 0.5 hr.$^{-1}$ in glucose plus acetate deficiency is attributed to incorporation of acetate without division and as previously mentioned, the amount of incorporation drops at D 0.1 hr.$^{-1}$ giving the recorded drop in cell size. Large cell mass and ovoid appearance is likewise typical of sulphur deficiency also suggesting that here there is incorporation of acetyl fragments. If acetyl oxidation is blocked, and the high concentrations of acetate in the medium do confirm this, some other cell mechanism allowing incorporation must also be affected at the low growth rates. The yield of dry weight per unit of ingoing sulphur is almost twice as high for this organism as that recorded by Postgate and Hunter (1962) for A. aerogenes which also suggests that synthesis of cell material is deranged.

It might be concluded that the cell weight reflects the sensitivity of certain cell mechanisms to particular nutrient deficiencies but that in general the growth rate is the determining factor.

3b. Nucleic Acids: DNA

The DNA content per cell and most probably the number of nuclei appears to be directly related to the cell size. At low specific growth rates in sulphur limitation and glucose limitation plus acetate, cell mass and DNA

content per cell is high. At low specific growth rates in nitrogen deficiency, cell mass and DNA content per cell is low. At high specific growth rates in all deficiencies the cell mass and DNA content per cell is high.

It is hardly surprising that the DNA content per cell and cell size are inter-related but the present experiments do not prove whether cell division or nuclear division is inhibited although intuitively we might decide on the former. In view of the conclusions from the paragraph on cell size, it is not difficult to understand why lack of sulphur or excess acetate might inhibit cellular division but it is more difficult to understand why cellular division is inhibited with increasing growth rate.

3b. Nucleic Acids: RNA

The content of RNA per cell appears (with the exception of sulphur deficiency) to be independent of the specific growth rate, the faster the cell is growing the greater the content of RNA. There are, of course, individual variations, the value of RNA found for low growth rates in nitrogen deficiency being less than half that of glucose and potassium deficiencies. However, in sulphur deficiency, and to a much lesser extent glucose limitation plus acetate, the mean content of RNA per cell is large at low growth rates indicating the the deficiency or environment is

affecting synthesis. Therefore, we might conclude that RNA synthesis and RNA content per cell are less affected by conditions of growth in the chemostat than DNA synthesis and DNA content per cell. The fact that increased growth rates have higher contents of RNA per cell are in good agreement with the findings of many authors, particularly Herbert (1958) and Schaechter et al. (1958).

3c. Possible storage compounds: Polysaccharide and Polyglucose

The results of the two nitrogen deficiencies clearly show that accumulation of polyglucose can occur in B. megaterium. The fact that the percentage of polysaccharide was higher and the percentage of polyglucose was lower in one run may be ascribed to the low phosphate content of the medium in Fermentor 1. The media of higher phosphate content will have a higher retention of carbon dioxide, provided pH values are similar. Palmstierna (1956) showed that in washed cells of E. coli B, in the absence of external carbon sources but carbon dioxide, moderate synthesis of polyglucose occurred at the expense of unknown components in the cell. Therefore, we might suppose that the carbon dioxide tension of the suspending medium can affect the rate of polyglucose synthesis. High

phosphate concentrations in the presence of excess glucose might also be expected to favour increased rates of phosphorylation and generally to increase levels of intermediates, such as glucose-1-phosphate, suitable for polyglucose synthesis.

It was also demonstrated that cells grown in nitrogen deficiency were capable of breaking down their polyglucose in washed suspensions in the presence of moderate concentrations of oxygen. The initial breakdown indicates that the phosphorylase which is responsible for the polyglucose breakdown is present during growth. Therefore, unless synthesis and breakdown are concomitant processes, feed-back inhibition may prevent breakdown. How then can the accumulation of polyglucose in nitrogen deficiency be explained in terms of Madsen's hypothesis (1963)? One would need to stipulate constant or increasing levels of UDPG with reduced growth rate, but this seems unreasonable in nitrogen deficiency. Constant or increasing levels of UDPG with reduced growth rate are also in opposition to the findings of Franzen and Binkley (1961) who analysed the distribution of different nucleotides occurring in E. coli cells growing at different rates and found ATP per unit mass was constant but GTP and UTP increased in amount

per unit mass with increasing growth rate. Another reason for an increased content of polyglucose might be due to an inhibition of phosphorylase, not necessarily by UDPG, but perhaps by a mass action effect of high levels of glucose-1-phosphate. To settle this matter more details of steady state levels of intermediates and enzymic activities are required, not only at different dilution rates but also in different deficiencies where glucose is in excess. Until cells grown at dilution rates well below D 0.1 $hr.^{-1}$ are investigated for polyglucose content, no hard conclusions can be drawn as the results of our experiments, and those of Holme and Palmstierna (1957), show a declining rate of synthesis at the lowest growth rates. One of the most disappointing features of the two Fermentors was that until recently is was impossible to obtain dilution rates below D 0.1 $hr.^{-1}$.

The study by Ghuysen (1960) on the composition of the cell wall of B. megaterium strain KM has shown that in cells grown aerobically in 5% peptone, approximately 6% of the dry weight of the walls consisted of a poly-glucose compound which was apparently not 'associated' with the teichoic acid - mucopeptide complex forming the main constituent of the cell wall. The presence of approximately 1.5% of the dry weight as a non-utilisable

polyglucose compound in glucose, potassium and sulphur limitations agrees with Ghuysen's findings; it suggests that intracellular accumulation in nitrogen deficiency of this polyglucose compound which is normally a cell wall component, is the result of direct impairment by low concentrations of nitrogen of the mechanism controlling synthesis. We might conclude that in B. megaterium the polyglucose compound is a normal structural component of the cell wall at all growth rates and that, except in nitrogen limitation, its synthesis has not been demonstratably affected by any chemical component of the environment or by the growth rate. These findings also support the doubts cast by Holme (1957) on the role as a storage compound of glycogen in E. coli. The fact that intracellular polyglucose compounds function as ideal storage compounds should not be mistaken to mean that this is the one and only reason for their presence, particularly as the present experiments demonstrate that accumulation is not a function of the growth rate but only a function, directly or indirectly, of the limiting concentration of nitrogen.

3c. Possible storage compounds: PHB

An important finding was the occurrence of PHB during four different carbon limitations at similar low growth rates when the limiting carbon concentration must

have been very low. These facts appear to fulfil the first condition in the definition of a storage compound (Wilkinson, 1959) that accumulation should occur when the supply of energy from exogenous sources is in excess. Energy sufficiency must increase the level of intermediates, most probably of acetyl fragments, to one sufficient to prime polymerisation of PHB at just above D 0.2 $hr.^{-1}$. The faster the growth rate, the higher the concentration of intermediates and the more acetyl fragments converted into PHB until the maximum rate of synthesis occurs at D 0.4 - 0.5 $hr.^{-1}$. Around this growth rate synthesis remains constant or drops slightly. However, in glucose deficiency increasing growth rate and increasing GOR values produce more acetyl fragments than can be converted into PHB and acetate appears in the medium.

No PHB was formed in glucose limitation plus acetate at D 0.2 - 0.5 $hr.^{-1}$, but large amounts of acetate were incorporated. The inability to form PHB might be explained by supposing that most of the energy or intermediates used in PHB synthesis at D 0.2 - 0.5 $hr.^{-1}$; are now used to incorporate acetate into cell material and that only at D 0.5 $hr.^{-1}$ is the GOR producing intermediates or energy faster than acetate incorporation. This

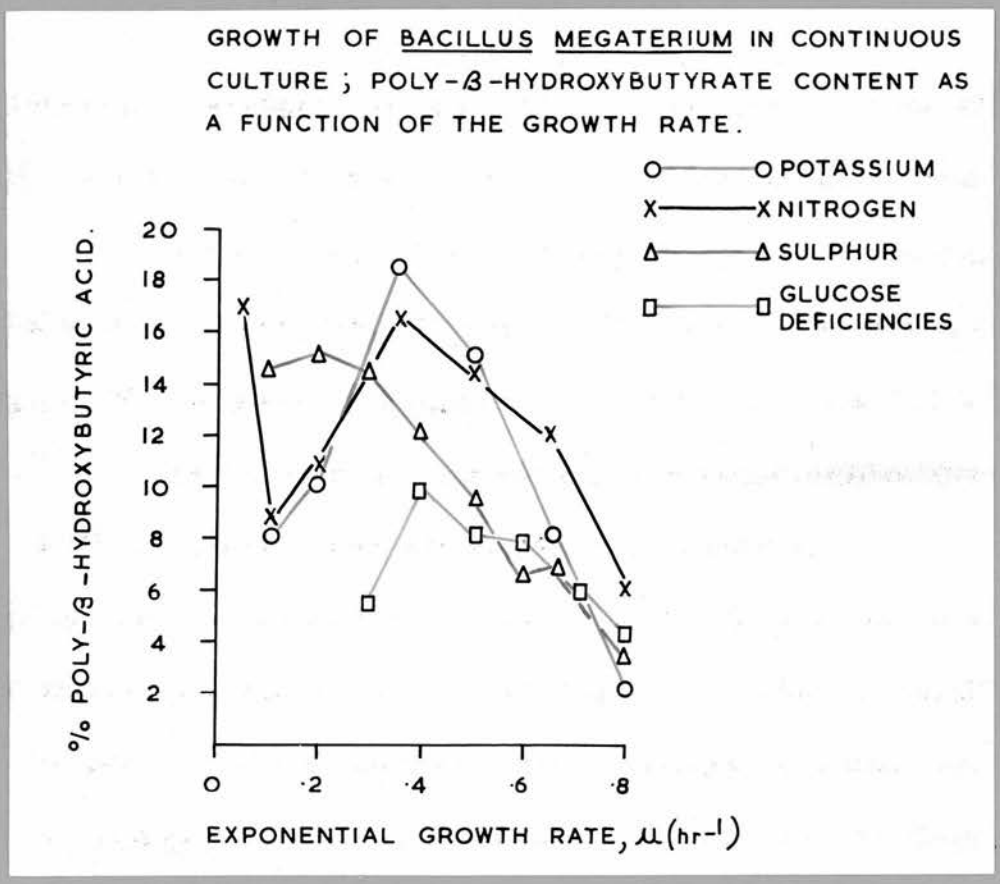

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE ; POLY-β-HYDROXYBUTYRATE CONTENT AS A FUNCTION OF THE GROWTH RATE.
POTASSIUM
NITROGEN
SULPHUR
GLUCOSE DEFICIENCIES
% POLY-β-HYDROXYBUTYRIC ACID.
20
18
16
14
12
10
8
6
4
2
0
·2
·4
·6
·8
EXPONENTIAL GROWTH RATE, μ(hr⁻¹)

Fig. 38.

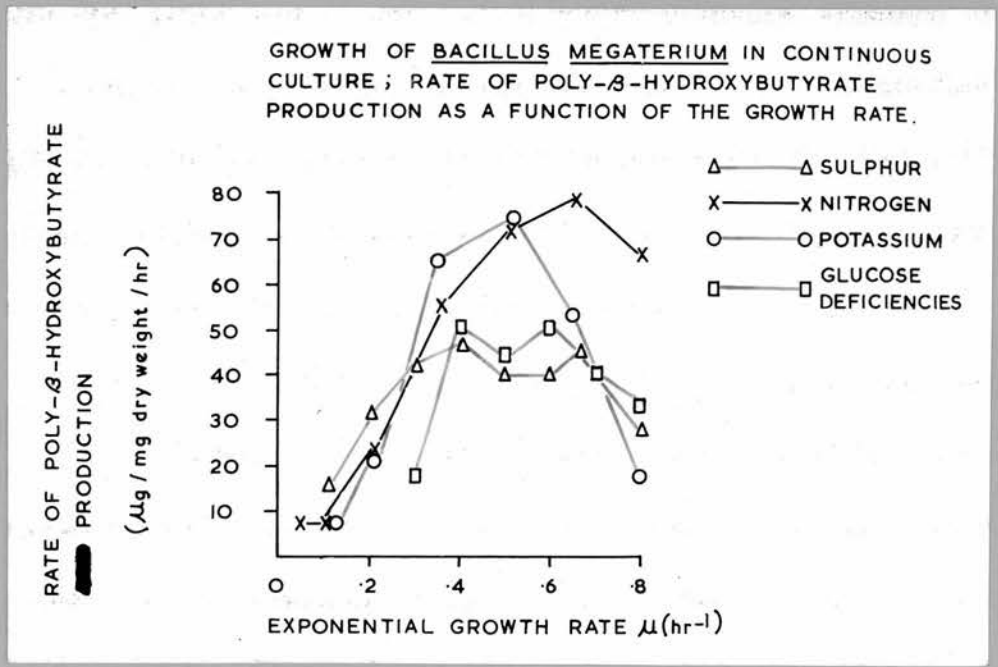

GROWTH OF BACILLUS MEGATERIUM IN CONTINUOUS CULTURE ; RATE OF POLY-β-HYDROXYBUTYRATE PRODUCTION AS A FUNCTION OF THE GROWTH RATE.
SULPHUR
NITROGEN
POTASSIUM
GLUCOSE DEFICIENCIES
RATE OF POLY-β-HYDROXYBUTYRATE PRODUCTION
(μg / mg dry weight / hr)
80
70
60
50
40
30
20
10
0
·2
·4
·6
·8
EXPONENTIAL GROWTH RATE μ(hr⁻¹)

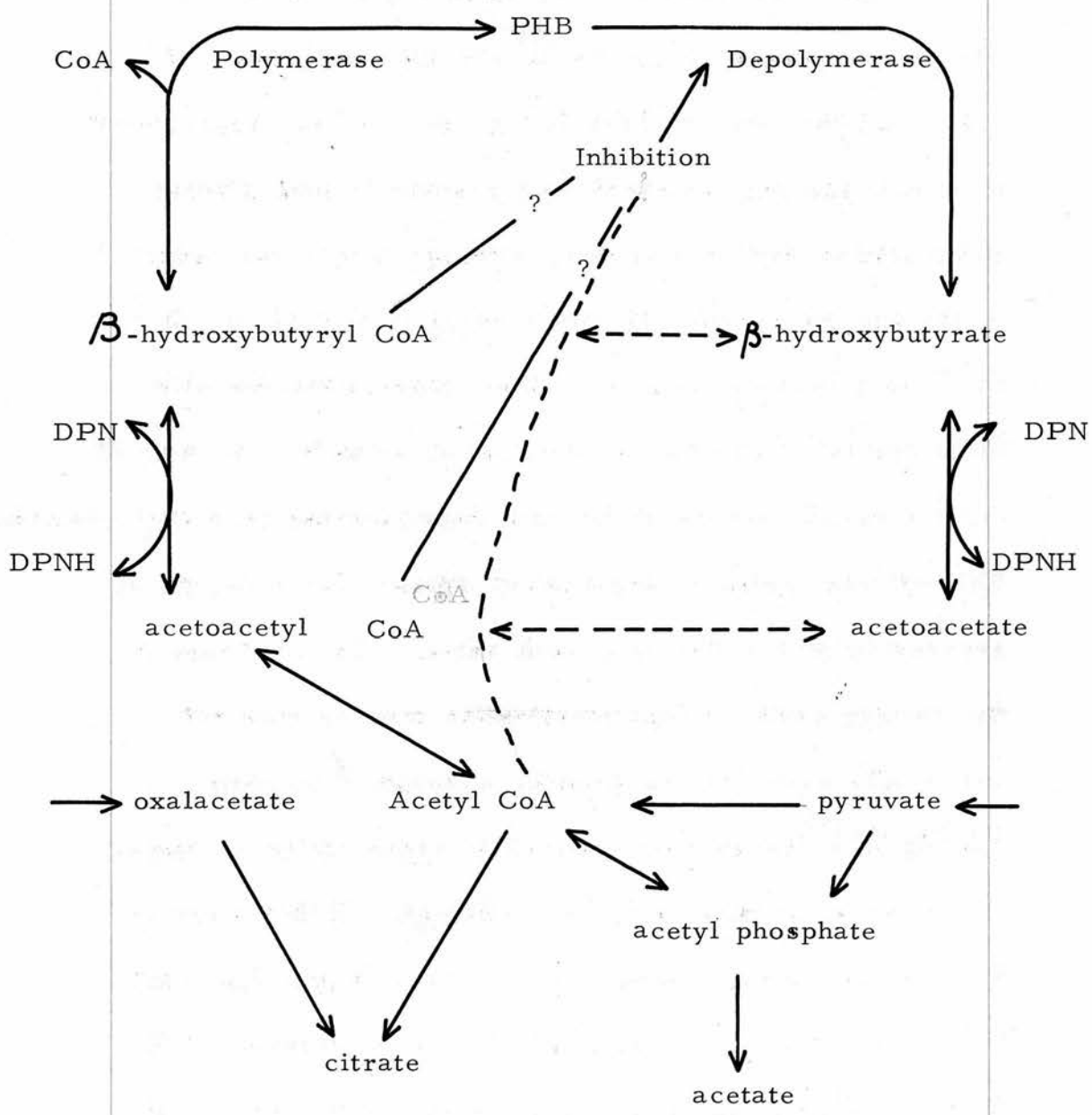
Fig. 39.

is not the only reason that might be suggested, especially as it does not explain why so little PHB was found at all growth rates in nitrogen deficiency plus acetate. If acetate does inhibit PHB formation why does it occur above D 0.5 $hr.^{-1}$ in glucose limitation plus acetate? No single explanation appears to cover all the known facts.

It is useful to compare the total content of PHB and the rate of PHB synthesis in different deficiencies and these are shown in Figs. 38 and 39. Generally speaking, the maximum content of PHB and the maximum rate of synthesis of PHB occur near D 0.4 $hr.^{-1}$, dropping in all growth limitations above D 0.6 $hr.^{-1}$. The rate of synthesis falls below D 0.4 $hr.^{-1}$ but in nitrogen limitation it remains constant below D 0.1 $hr.^{-1}$ this being the only deficiency where very low growth rates were obtained. The rate of synthesis of PHB in sulphur limitation is higher between D 0.1 - 0.3 $hr.^{-1}$ and results in a higher proportion of the dry weight occurring as PHB, but it should be remembered that the cells were unduly large during this period and the maximum rate of synthesis of PHB was even lower than in glucose limitation. The evidence shows that the rate of synthesis is qualitatively affected by the growth rate, and quantitatively by the level of inter-

Fig. 40

Possible pathways in the cellular control of PHB levels



mediates, which in turn is related to the concentration of the limiting nutrient.

The depolymerisation experiments are of some interest in understanding the underlying mechanisms of control of the level of PHB in the cell. These experiments show that the depolymerase was present in both glucose and nitrogen deficiencies and, although it was not recorded in the results section, it was present in D 0.35 and D 0.5 $hr.^{-1}$ in potassium deficiency also (these were the only rates tested). The immediate depolymerisation on removal from a carbon source shows that depolymerase is always present. The different rates of degradation suggest some degree of repression with different growth rates. As mentioned in the results section, depolymerisation may or may not continually occur during growth, although if cellular mechanisms are generally aimed at conservation of energy the latter suggestion is to be preferred. If this were not so, the high energy sulphydryl groups of acetyl CoA will be dissipated by continual synthesis and breakdown of PHB. If the depolymerase is inhibited during growth as a means of preventing energy dissipation, then some feed-back control mechanism is likely involving perhaps β-hydroxybutyryl Co A, as depicted in Fig. 40, or even acetoacetyl

Co A or acetyl Co A. Wilkinson (unpublished results) has shown that in extracellular extracts of active granules, β-hydroxybutyrate, acetoacetate, and acetate cause denaturation of the depolymerase. Merrick and Doudoroff (1961) showed that in extracellular extracts of R. rubrum polymerisation of β-hydroxybutyryl Co A occurred, but an active depolymerase caused concurrent breakdown as well. In washed cell suspensions of R. rubrum it was demonstrated by Stanier et al. (1959) that breakdown of PHB did not occur in a reducing atmosphere, indicating feedback control of depolymerisation, but breakdown did occur as soon as carbon dioxide was added. B. megaterium on the other hand will depolymerise PHB anaerobically to produce β-hydroxybutyrate and acetoacetate (Macrae & Wilkinson 1958b). If in these anaerobic experiments of Macrae and Wilkinson the cells were unable to form the inhibitor of depolymerase, that is one of the Co A derivatives previously mentioned, then depolymerisation would occur with production of the observed products.

The picture presented by the occurrence of PHB is extremely complex and apparently differs from organism to organism. The presence of synthetic and degradative systems for PHB at practically all growth rates and under all nutrient limitations suggests that its occurrence is of

prime importance to the cell. However, the advantages gained by having these mechanisms of synthesis and degradation in cells which have been growing vegetively for many weeks, has not been demonstrated. It might be claimed that cells which had lost their ability to synthesise PHB would have an advantage in a selective system like the chemostat and might predominate more or less rapidly, but such was not the case. Indeed, using Sudan Black staining at each dilution rate in every nutrient limitation, it was difficult to find one cell which did not stain. Therefore, it might be fair to conclude that the occurrence of the complex mechanisms involved in maintaining PHB in growing cells had some other function besides carbon storage, perhaps more closely associated with growth than starvation. Finally, it must be mentioned that KM is as asporogenous variant of B. megaterium which is normally a sporing organism. Many claims (Knaysi, 1945 and Tinelli, 1955 a, b) have been made that PHB is a reserve to assist in sporulation, but recent trends (Slepecky and Law, 1961, Nakata, 1962) conclude that, while PHB does supply energy and carbon for sporulation, it is not essential. PHB is known to occur in many non-sporing bacteria and its occurrence would seem to have, as the results presented show, a more general significance than as an aid to sporulation.

Control of Glucose Oxidation and Production of Overflow Compounds

All the deficiency limitations show that the GOR increases with increasing growth rate. Nitrogen and potassium limitations show almost similar rates at all D values and perhaps we might infer that these values are a reflection of the growth rate. Sulphur limitation is similar except tat the lowest growth rates where the low GOR is probably a specific effect due to sulphur shortage.

Therefore, in all the runs where glucose was in excess, there was an interdependence of growth rate and GOR between D 0.2 - 0.8 $hr.^{-1}$. The interdependence suggests that glucose catabolism is controlled to suit the anabolic requirements of the cell, which is the reverse of the findings of Rosenberger and Elsden (1960) who showed that in an anaerobic, continuously growing culture of Strep. faecalis, the rate of glucose utilisation was independent of the growth rate. This finding in B. megaterium suggests that enzymic levels, including permeases, are strongly suppressed at lower growth rates. The GYC figures do show that progressively more glucose is catabolised at lower growth rates when glucose is in excess than in glucose limitation. Supporting this claim that the growth rate determines to a great extent not only the RNA,

DNA and cell size, Chaux and Petit (1957) found that the cytochrome absorbtion intensity of cells of B. subtilis, grown at fast and slow growth rates in a simple chemostat, varied according to the growth rate.

Other authors (Hanson, Scrinivasan and Halvorson, 1961, 1963 a & b) have demonstrated that in B. cereus strain T the activities of many TCA cycle enzymes, including oxalacetate - acetyl Co A condensing enzyme, aconitase, succinic dehydrogenase, fumarase and malic dehydrogenase, were nearly ten times lower in fast growing vegetative cells than in very slow growing cells not irreversably committed to sporulation. B. cereus is very like B.megaterium in that on glucose medium, acetic acid is produced during fast vegetative growth. Admittedly, in the experiments by the above authors the slow growing cells were starting to oxidise some of the previously produced acetate and some induction of the TCA cycle enzymes would have occurred. However, the general low activities of the TCA cycle enzymes, and in particular the low level of condensing enzyme activity since it controls acetyl Co A entry into the TCA cycle, indicates that during fast growth when there is accumulation of acetate the TCA cycle is favouring anabolic requirements. Therefore, the oxidative role of the TCA cycle is diminished at faster growth rates

because of these large anabolic requirements. In the glucose limited run we see that acetate was produced in increasing amount from D 0.5 $hr.^{-1}$. Similar observations have been made on yeasts (Maxon and Johnson, 1953) and A. cloacae (Pirt, 1957). Under carbon limitation in continuous culture both these organisms produce acetic acid at growth rates somewhat below their maximum, production occurring in A. cloacae at nearly similar D values to those reported here. It seems that the TCA cycle deals with the increasing rate of catabolism of glucose up to D 0.5 but is unable to handle the products above this point, suggesting increased activity of systems leading to acetyl Co A and constant or decreasing TCA cycle enzyme activities.

In potassium limitation the constant rate of acetate production from D 0.2 - 0.8 $hr.^{-1}$ nearly accounts for all the glucose utilised suggesting either impairment of the oxidative TCA cycle or, much more likely, that acetate production is the normal cell mechanism for production of energy from glucose. After all, cells growing in a complete medium produce almost stoichiometric amounts of acetate and pyruvate (Hanson et al. 1963a). Oxoglutarate production, which might be used as a measure of the effectiveness of acetate entry into the TCA cycle, is low in potassium deficiency.

Low GOR values in sulphur deficiency would cut the acetate production rate and this indeed happened. Again oxoglutarate production is low. Therefore, in sulphur and potassium deficiency the oxidative function of the TCA cycle is apparently low.

In nitrogen deficiency acetate production is low for the high GOR value while oxoglutarate production is much higher than in sulphur or potassium limitation. Until it is certain no other products are produced it is not clear if all the glucose metabolised is produced as carbon dioxide but the higher oxoglutarate production suggests much greater handling of acetyl fragments by the TCA cycle. If the low nitrogen concentration does damage a controlling mechanism, as is suggested in polyglucose synthesis, then the oxidative mechanism of the TCA cycle may well be released.

SUMMARY

SUMMARY

1. Two single stage chemostats have been constructed and have been shown to operate satisfactorily in the range D 0.1 - 0.8 $hr.^{-1}$.

2. Bacillus megaterium has been grown continuously using the following limiting nutrients: carbon and energy including glucose, glucose plus acetate, glycerol, lactate or succinate and also nitrogen, sulphur or potassium.

3. In all limitations except glucose plus acetate it has been shown that selection of mutants capable of faster growth rates than the parent occurred.

4. In glucose limitation there was selection of slower growing strains corresponding to each growth rate tested. This was less obvious in nitrogen limitation.

5. It has been found that when the limiting nutrient is held at a constant level in the inflowing medium the amount of growth (dry weight per ml.) and output of dry weight of cells varies with the growth rate in a manner characteristic of the limiting nutrient. It was concluded that the kinetics of the mechanisms involving uptake and utilisation may be substantially different in each limitation.

6. The Gram-variable properties of this organism

can be a function of the specific growth rate. It was concluded that the slower growing cells had a thinner or more porous mucopeptide coat than the faster growing cells.

7. The cell mass has been found to be generally a function of the growth rate with certain specific exceptions; in glucose limitation the cells are smaller at faster growth rates than in other limitations while below D 0.5 $hr.^{-1}$ the cells are unduly large in sulphur limitation and glucose limitation plus acetate.

8. The DNA content per cell was shown to be a function of the growth rate at fast doubling times but at decreasing growth rates the limiting nutrient played an increasing role on the content of DNA, particularly those limitations which affect the cell mass.

9. The RNA content per cell was also shown to be a function of the growth rate except in:- (a) nitrogen limited cells at D 0.1 and D 0.2 $hr.^{-1}$ where lower than expected levels of RNA were produced, (b) in sulphur limited cells at D 0.1 and 0.2 $hr.^{-1}$ where RNA levels were approximately twice those in other limitations.

10. Very little polyphosphate was detected even in sulphur deficiency at low growth rates where batch culture experiments suggest accumulation should occur.

The possible reasons for this lack of polyphosphate accumulation are discussed.

11. Extensive polyglucose accumulation only occurred in nitrogen limitation at lower growth rates; the accumulated polyglucose was shown to be broken down in washed cell suspensions lacking a carbon source. The presence of a small polyglucose level in all other deficiencies was presumed to be a constituent of the cell wall; it was not broken down by washed cell suspensions lacking a carbon source.

12. It is suggested that nitrogen deficiency impairs a controlling mechanism in polyglucose synthesis, the accumulation of polyglucose resulting.

13. The extensive occurrence of PHB in all carbon limitations, even at fairly low growth rates, fulfils one criterion in the definition of a storage compound that it can be stored even under moderate carbon limiting conditions.

14. The occurrence of a similar pattern of levels of PHB in all growth deficiencies suggests that the growth rate is of considerable importance.

15. PHB depolymerase was present in all samples examined.

16. The last three points suggest that PHB is a true storage compound because its occurrence is apparently only dependent on the intracellular level of an intermediate whose regulation is apparently controlled largely by the growth rate and not the nature of the carbon source or the limiting nutrient.

17. The rate of glucose breakdown was found to be strictly controlled by the growth rate in all deficiencies where glucose was in excess.

18. The production of three extracellular products was followed, namely acetic acid, pyruvic acid and 2-oxoglutaric acid. The production rate of each was largely independent of the limiting nutrient at faster growth rates but as the doubling time lengthened the production rate of each became more dependent on the limiting nutrient in question.

BIBLIOGRAPHY

Allen, R.J.L.	1940	Biochem. J. 34, 858.
Atwood, K.C., Schneider, L.K. & Ryan, F.J.	1951	Proc. natl. Acad. Sci. U.S. 37, 146.
Barry, C., Gavard, R., Milhaud, G., & Aubert, J.D.	1953	Ann. Inst. Pasteur, 84, 605.
Bernheimer, A.A. ..	1953	J. exp. Med. 97, 591.
Bryson, V.	1952	Science, 116, 48.
Burdon, K.L., Stokes, J.C. & Kimbrough, C.E.	1942	J. Bact. 43, 717.
Burton, K.L.	1946	J. Bact. 52, 665.
Burton, K.	1956	Biochem. J. 62, 315.
Callow, D.S. & Pirt, S.J.	1956	J. gen. Microbiol. 14, 661.
Callow, D.S. & Pirt, S.J.	1961	J. appl. Bact. 24, 12.
Cavallini, D. & Frontali, N.	1954	Biochem. biophys. Acta, 13, 439.
Chaix, P. & Petit, J.F.	1957	ibid., 25, 481.
Cocito, C. & Bryson, V.	1958	Bact. Proc. 38.
Cocito, C. & Vogel, Jr., H.J.	1958	Proc. Xth. int. Cong. Genetics, Montreal.
Collins, J.F. & Kornberg, H.L.	1960	Biochem. J. 77, 430.
Contois, D.E.	1959	J. gen. Microbiol. 21, 40.
Cooper, C.M., Fernstrom, G.A. & Miller, S.A.	1944	Indust. Engng. Chem. (Ind.) 36, 504.

Dawes, E.A. & Holms, W.H.	1958	Biochim. biophys. Acta, 30, 278.
Dawes, E.A. & Ribbons, D.W.	1962	Annu. Rev. Microbiol. 16, 241.
Dawson, P.S.S. ..	1960	J. Biochem. Microbiol. Technol. Eng. 2, 227.
Denbigh, K.G.	1944	Trans. Faraday Soc. 40, 352.
Denbigh, K.G.	1951	The Thermodynamics of the Steady State. Methuen, London.
Dische, Z.	1930	Mikrochemie, 8, 4.
Doudoroff, M. & Stanier, R.Y.	1959	Nature, Lond. 183, 1440.
Dreywood, R.	1946	Indust. Engng. Chem. (Anal.) Edit. 18, 499.
Elsworth, R., Meakin, L.R.P., Pirt, S.J. & Capell, G.H.	1956	J. appl. Bact. 19, 264.
Elsworth, R., Williams, V. & Harris-Smith, R.	1957	J. appl. Chem. 7, 261.
Elsworth, R., Capell, G.H. & Telling, R.C.	1958	J. appl. Bact. 21, 80.
Fairbairn, N.J. ..	1953	Chem. & Ind. p. 86.
Finn, R.K. & Nowry, J.E.	1959	Appl. Microbiol. 7, 29.
Franzen, J.S. & Binkley, S.B.	1961	J. biol. Chem. 236, 515 (66).
Friedmann, T.E. ..	1938	ibid. 123, 161.
Formal, S.B., Baron, L.S. & Spilman, W.	1956	J. Bact. 72, 168.

Forsythe, W.G.C., .. Hayward, A.C., & Roberts, J.B.	1958	Nature, Lond. 182, 800.
Foster, J.W.	1947	Bact. Rev. 11, 167.
Fuld, G.J., Mateles, R.I., & Kusmeirek, B.W.	1961	In 'Continuous Culture of Microorganisms' p. 54 Soc. Chem. Ind. Monograph No. 12. London.
Ghuysen, J.M.	1961	Biochem. biophys. Acta, 50, 413.
Good, C.A., Kramer, H., & Somogyi, M.	1933	J. Biol. Chem. 100, 485.
Gorini, L.	1960	Proc. natl. Acad. Sci. U.S. 46, 682.
Hanson, R.S., Srinivasan, V.R., & Halvorson, H.O.	1961	Biochem. Biophys. Res. Commun. 5, 457.
Hanson, R.S., Srinivasan, V.R., & Halvorson, H.O.	1963a	J. Bact. 85, 451.
Hanson, R.S., Srinivasan, V.R., & Halvorson, H.O.	1963b	J. Bact. 86, 45.
Harold, F.M.	1960	Biochim. biophys. Acta, 45, 172.
Harold, F.M.	1962	J. Bact. 83, 1047.
Harold, F.M.	1963	ibid, 86, 216
Harold, F.M. & Sylvan, S.	1963	ibid, 86, 222
Hartman, T.L.	1940	Stain Technol. 15, 23.
Herbert, D., Elsworth, R., & Telling, R.C.	1956	J. gen. Microbiol. 14, 601.

Herbert, D.	1958	In 'Recent Progress in Microbiology', p. 381, Symp. 7th int. Congr. Microbiol.
Herbert, D.	1961	In 'Continuous Culture of Microorganisms' p. 21. Soc. Chem. Ind. Monograph No. 12, London.
Holme, T.	1957	Acta chem. scand. 11, 763.
Holme, T. & Edebo, L.	1961	Acta path. microbiol. scand. 54, 164.
Holme, T. & Palmstierna, H.	1955	Acta chem. scand. 9, 1021.
Holme, T. & Palmstierna, H.	1956a	ibid. 10, 553.
Holme, T. & Palmstierna, H.	1956b	ibid. 10, 578.
Holme, T. & Palmstierna, H.	1956c	ibid. 10, 155.
Hough, L. & Jones, J.K.N. & Wadman, W.H.	1950	J. chem. Soc. 1702
Hugget, A. St. G., & Nixon, D.A.	1956	Biochem. J. 66, 12P
Jacob, F., Schaeffer, P. & Wollman, E.L.	1960	In 'Microbiol Genetics' p. 88. 10th Symp. Soc. gen. Microbiol.
Jermyn, M.A. & Isherwood, F.A. ..	1949	Biochem. J. 44, 402.

Jerusalimskij, N.D. ..	1958	In 'Continuous Cultivation of Micro-organisms', Prague, Symposium, p. 53. Ed. I. Malek.
Juni, E., Kamen, M.D., Reiner, J.M. & Spiegelman, S.	1948	Arch. Biochem. 18, 387.
Kabat, E.A., & Meyer, M.M.	1961	In 'Experimental Immunology'; 2nd ed. p. 551. Thomas, Illinois.
Kepès, A., & .. .. Péud-Lenoël, C.	1952	Compt. Rend. Paris 234, 756.
Kjeldgaard, N.O., Maaløe, O. & Schaechter, M.	1958	J. gen. Microbiol. 19, 607.
Knaysi, G.	1945	Science, 102, 424.
Koepsel, H.J. & Sharp, E.S.	1952	Arch. Biochem. Biophys. 38, 443.
Kornberg, A. Kornberg, S.R. & Simms, E.S.	1956	Biochim. biophys Acta 26, 215.
Kornberg, H.L., Phizackerly, P.J.R., & Sadler, N.	1960	Biochem. J. 77, 438.
Kornberg, S.R. ..	1957	Biochim. biophys. Acta, 26, 294.
Law, J.H., & Slepecky, R.A.	1961	J. Bact. 82, 33.
Laybourne, R.L. ..	1924	J. am. med. Assoc. 83, 121.
Lemoigne, M., Delaporte, B., & Croson, M.	1944	Ann. Inst. Pasteur, 70, 224.
Maaløe, O., & Richmond, M.H.	1962	J. gen. Microbiol. 27, 269.

Mackie, T.J. & McCartney, J.E. .. .. .. In 'Handbook of Practical Bacteriology'. p. 112. 10th ed. Edinburgh & London. 1960.

Macrae, R.M. & Wilkinson, J.F. .. .. 1958a Proc. Roy. Phys. Soc. (Edinburgh) 27, 73.

Macrae, R.M. & Wilkinson, J.F. .. .. 1958b J. gen. Microbiol. 19, 210.

Madsen, N.B. .. .. 1961 Biochim. biophys. Acta, 50, 194.

Madsen, N.B. .. .. 1963 Canad. J. Biochem. 41, 561.

Malek, I. .. .. 1958 In 'Continuous Cultivation of Microorganisms'; Prague Symposium p. 11 Ed. I Malek.

Maxon, W.D. & Johnson, M.J. .. .. 1953 Indust. Engng. Chem. 45, 2554.

Merrick, J.M. & Doudoroff, M. .. .. 1961 Nature, Lond. 189, 890.

McQuillen, K. .. .. 1955 Biochim. biophys. Acta, 17, 382.

Monod, J. .. .. 1950 Ann. Inst. Pasteur, 79, 390.

Myers, J. & Clark, L.B. 1945 J. gen. Physiol. 28, 103.

Nakata, H.M. .. .. 1962 Bact. Proc. p. 47.

Nakata, H.M. & Halvorson, N.O. .. .. 1960 J. Bact. 80, 801.

Novick, A. & Szilard, L. 1950a Science, 112, 715.

Novick, A. & Szilard, L. 1950b Proc. natl. Acad. Sci. U.S. 36, 708.

Palmstierna, H.	1956	Acta Chem. scand. 10, 567.
Pirt, S.J.	1957	J. gen. Microbiol. 16, 59.
Pirt, S.J., & Callow D.S.	1959	Nature, Lond. 184, 307.
Pirt, S.J., & Callow, D.S.	1961	Sci. Repts. 1st Super. Sanita, I, 250.
Pirt, S.J., Thackeray, E.J., & Harris-Smith, R.	1961	J. gen. Microbiol. 25, 119.
Postgate, J.R., & Hunter, J.R.	1962	ibid. 29, 233.
Powell, E.O.	1956	ibid. 15, 492.
Powell, E.O.	1958	ibid. 18, 382.
Ribbons, D.W., & Dawes, E.A.	1963	Ann. N.Y. Acad. Sci. 102, 564.
Richmond, M.H., & Maaløe, O. ..	1962	J. gen. Microbiol. 27, 285.
Rogers, L.A., & Whittier, E.O. ..	1930	J. Bact. 20, 127.
Rosenberger, R.F. & Elsden, S.R.	1960	J. gen. Microbiol. 22, 726.
Rosenberger, R.F. & Kogut, M.	1958	ibid. 19, 228.
Salton, M.R.T. ..	1962	ibid. 30, 223.
Scandrett, F.J. ..	1953	Analyst, 78, 734.
Schaechter, M., Maaløe, O. & Kjeldgaard, N.O.	1958	J. gen. Microbiol. 19, 592.
Schlegel, H.G., Gottshalk, G., & Von Bartha, R. ..	1961	Nature, Lond. 191, 463.

Schneider, W.C. ..	1945	J. Biol. Chem. 161, 293.
Sierra, G., & Gibbons, N.E.	1962a	Canad. J. Microbiol. 8, 249.
Sierra, G., & Gibbons, N.E.	1962b	ibid. 8, 252.
Slade, H.D., Doughty, C.C. & Slamp, W.C.	1954	Arch. Biochem. Biophys. 48, 338.
Slepecky, R.A. & Law, J.H.	1960	Anal. Chem. 32, 1697.
Slepecky, R.A. & Law, J.H.	1961	J. Bact. 82, 37.
Smith, I.W., ... Wilkinson, J.F., & Duguid, J.P.	1954	ibid. 68, 450.
Stanier, R.Y., Doudoroff, M., Kunisawa, R., & Contopoulou, R.	1959	Proc. natl. Acad. Sci. U.S. 45, 1246.
Strange, R.E., Dark, F.A. & Ness, A.G.	1961	J. gen. Microbiol. 25, 61.
Tinelli, R.	1955a	Ann. Inst. Pasteur, 88, 364.
Tinelli, R.	1955b	ibid. 88, 642.
Unger, E.D., Stark, W.H., Scalf, R.E., & Kolachor, P.J.	1942	Indust. Engng. Chem. 34, 1402.
Weibull, C.	1953a	J. Bact. 66, 688.
Weibull, C.	1953b	ibid. 66, 696.
Wilkinson, J.F. ..	1959	Exp. Cell Research, suppl. 7, 111.
Wilkinson, J.F., Duguid, J.D., & Edmunds, P.N.	1954	J. gen. Microbiol. 11, 59.

Williamson, D.H.	..	1956	Ph.D. thesis, Edinburgh University
Williamson, D.H. & Wilkinson, J.F.	..	1958	J. gen. Microbiol. 19, 198.

APPENDIX

1.	Accles & Pollock Ltd.,	Stainless Tube Division, Oldbury, Birmingham.
2.	George Angus & Co. Ltd.,	Oil Seal Division, Coast Road, Wallsend, Northumberland.
3.	C.F. Boehringer & Sochne GmbH,	Mannheim, West Germany.
4.	Brown Brothers & Co. Ltd.,	Rosebank Iron Works, Edinburgh, 7.
5.	D.S. Crawford Ltd.,	29 Frederick Street, Edinburgh, 2.
6.	Richard Dalemann Ltd.,	325-327 Latimer Road, London, W.10.
7.	Dowty Seals Ltd.,	Ashchurch, Tewkesbury, Glos.
8.	J. & J.A. Dunn,	15 Blair Street, Edinburgh, 1.
9.	Esco (Rubber) Ltd.,	34-36 Somerford Grove, London, N.16.
10.	J.H. Fenner & Co. Ltd.,	98 Holm Street, Glasgow, C.2.
11.	Fibreglass Ltd.,	Ravenhead, St. Helens, Lancs.
12.	Fielden Electronics Ltd.,	Wythenshawe, Manchester.
13.	Firth-Vickers, Ltd.,	Staybrite Works, Weedon Street, Sheffield, 9.
14.	Fischer & Porter Ltd.,	50 Wellington Street, Glasgow, C.2.

15. Fry's Metal Foundaries Ltd.,	110, John Street, Glasgow.
16. Gittings & Hills Ltd.,	Tower Varnish Works, Long Acre, Birmingham, 6.
17. V.A. Howe & Co. Ltd.,	46 Pembridge Road, London, W.11.
18. Londex Ltd.,	Anerley Works, 207 Anerly Road, London, S.E. 20.
19. Midland Silicones Ltd.,	75 St. George's Place, Glasgow, C.2.
20. James Niel & Co. Ltd.,	Technical Service Dept., Sheffield.
21. Q.V.F. Ltd.,	Duke Street, Fenton, Stoke-on-Trent, Staffs.
22. Parvalux Ltd.,	Wallisdown Road, Bournemouth, Hampshire.
23. Power Flexible Tubing Co. Ltd.,	Derby Works, Vale Road, London, N.4.
24. W.G. Pye & Co. Ltd.,	Granta Works, P.O. Box 60, Cambridge.
25. J.W. Roberts Ltd.,	Chorley New Road, Horwich, Bolton.
26. Sangamo Weston Ltd.,	Enfield, Middlesex.
27. Suffolk Iron Foundary Ltd.,	Sifbronze Works, Stowmarket, Suffolk.

28. Sunvic Controls,	AEI Instrument Division, P.O. Box 1, Harlow.
29. Teddington Industrial Equipment Ltd.,	Sunbury-on-Thames, Middlesex.
30. Townson & Mercer Ltd.,	Croydon.
31. James Walker & Co. Ltd.	96a Constitution Street, Leith, Edinburgh.
32. J. & F. Wishart Ltd.,	18 Picardy Place, Edinburgh, 1.
33. G.H. Zeal Ltd.,	Lombard Road, Morden Road, Merton, London, S.W.19.

Manufactures Products and details of their use

1. Stainless Steel Tube, any length, bore and wall thickness can be ordered. Normally all tubes are supplied polished on the outside, and descaled but not polished inside.

2. 'Gaco' Metal Insert type shaft oil seals. Ref. MIS 04 ($\frac{1}{4}$ in. shafts). These oil seals should be smeared liberally with silicone grease (MS 44 Ref. 19) and packed between two felt washers impregnated with grease.

3. ATP, ADP, Glucose oxidase kit, and Tris buffer.

4. Stainless Steel Sheet. This company will supply on request BSS En 58C stainless steel sheet of various sizes being the scrap cuttings from ships stabiliser fins. This material has marked corrosion resistance but is very tough to machine.

5. Tartan Shortbread Biscuit Tins. 8 in. diam. by $3\frac{1}{2}$ in. deep.

6. White perspex sheet.

7. Dowty Bonded Seals. $\frac{1}{4}$ in. BSP for the ports on the Fermentor top. $\frac{3}{4}$ in. BSP for the stirrer gland in Fermentor 1.

8. Tufnol Sheet. Aluminium Sheet.

9. Silicone Rubber Tubing, various sizes. Wall of 1 m.m. thickness is enough for ordinary purposes but tubing of wall thickness 2 m.m. should be used in the peristaltic pump (Ref. 17).

10. Resilla Bond Flexible Couplings for joining the stirrer shaft of Fermentor 1 to the stirrer motor. Coupling No. 0.

11. Unbonded '2A' Fibreglass suitable for bacteriological filters. This material is packed in the biscuit tins and has been so satisfactory that it has never been replaced.

12. Precision Temperature Controller, type TcF2 which can be operated though a Sunvic relay, type F 202/6, if the load is greater than 2 amps on either of the contacts of the Controller. Servograph, Mark II, 4 point recorder.

13. Stainless Steel Rod. Order BSS En58M. This grade of stainless steel is particularly suitable for machining in general and screw-cutting in particular. The capital M stands for free machining grade. Grades En58A, B, or C, are also suitable but less easy to work.

14. Direct scaled tubes for metering air in the range 50 - 960 ml. of air per min. at 18°, $4\frac{1}{2}$ in. W.G., were used.

15. Stainless Steel Solder and Flux. Care should be taken to present as little of the solder surface as possible to the culture of growing organisms as it contains toxic metals.

16. Rochard 444/232 epoxy-resin and thinner 444/275. The clean dry magnet is dipped in the resin and allowed to dry at 180° in a hot air oven. When sufficiently dry it is dipped again and the process repeated 2 or 3 times until a sufficiently thick coat is built up round the magnet. Finally it is baked for 12 - 16 hr. at 180°.

17. Sigmamotor Model T-8 peristaltic pump.
Zero-Max Gearchanger Model 14 with screw control.
Zero-Max Gearchanger Model E with microdial screw control.

18. Auto-Resetting IMP Timer. Maximum range 1 hr.

19. MS 44 Bearing grease (8 oz.). This is a suitable lubricant for the gland in Fermentor 1 as it does not flow on autoclaving.

20. Eclipse Power Magnets 812B and 813C. The driving magnet on the stirrer motor is 813C and the driven magnet in the vessel (Fig. 23) is 812B machined to the dimensions shown.

21. Fermentor Vessels as shown in Fig. 4. QV Ref. 005044 RCW/05 File No. S40013.

22. Fractional Horsepower Motors, specify continuously rated. Pump motors are Type SD 14 with a single reduction unit, either 400 r.p.m. or 560 r.p.m. is suitable. Stirrer

Motors are type SD 13. Fermentor 1; - 1400 r.p.m. Fermentor 2; - 920 r.p.m.

23. Stainless Steel BOA Super-Metalflex tubing, 8 ft. 8 in. long $\frac{3}{4}$ in. bore, with standard corrugations, fitted at each end with 4 in. length of $\frac{1}{2}$ in. O.D. 22-guage stainless steel pipe. This length has proved suitable in 10 l. aspirators.

24. Dynacap pH Meter. Autotitrator Controller No. 11602 Delivery Unit No. 11610. Ingold Single Rod type 405 pH Electrode.

25. Ferobestos Solid Rod - Specify LF 37. LF signifies fine weave, 3 signifies colloidal graphite impregnation, 7 signifies a special stabilisation process to remove free phenol and formaldehyde.

26. Sangamo Resistance Bulb, type SG 110G/4/215, 8 in. long.

27. Special Sifbronze 22% Nickel Rods ($1/_8$ in.) plus Sifbronze Flux for brazing stainless steel.

28. Nullmatic Pressure Regulator, model 41/30, plus air filter model 2306, and preferably also fitted with a pressure gauge on the reduced pressure line.

29. YF Solenoid Valve HI. Model A.

30. Laboratory Refrigeration Unit, Minus Twenty Model, plus abath containing antifreeze and pump motor. Picien Wax.

31. Neoprene sheet $1/8$ in. thick. Shore hardness 60. Gaskets are cut from this sheet to make a seal between the fermentor and the stainless steel top plate.

32. Dexion angle-iron strip.

33. Electric Contact Thermometer, with four operating contacts at 25°, 30°, 35°, 37°. It is 8 in. in length $7/32$ in. diameter. Zecol Plug-in Contact Relay unit for operating with the contact thermometer.